Trypanocidal drugs and the problem of drug resistance in West Africa

Hervé Sèna Vitouley

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Promoters:

Prof. Dr. Pierre Dorny

Prof. Dr. Jan Van Den Abbeele

Dr. Vincent Delespaux

Dr. Zakaria Bengaly

Ghent University,

Faculty of Veterinary Medicine,

Department of Virology, Parasitology and Immunology

"Jésus-Christ est mon rempart et mon salut"

This thesis is dedicated to the Blessed Virgin Mary and to my beloved daughter Victoria.

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List of Abbreviations

AAT: Animal African Trypanosomosis

ABC: Adenosine tri-phosphate Binding Cassette

Ab-ELISA: Antibody Enzyme Linked Immuno-Sorbent Assay

AFLP: Amplified Fragment Length Polymorphism

Ag-ELISA: Antigen Enzyme Linked Immuno-Sorbent Assay

BCT: Buffy Coat Technique

bp: base pair

CATT: Card Agglutination Test for Trypanosomiasis

CI: Confidence Interval

CIRDES: Centre International de Recherche-Développement sur l'Elevage en zone Subhumide

DA: Diminazene Aceturate

DIGIT: Drug Incubation Glossina Infectivity Test

DIIT: Drug Incubation Infectivity Test

DMSO: Dimethyl Sulfoxyde

DNA: Desoxyribonucleic Acid

FAO: Food and Agriculture Organization

FQE: Fluoroquinolone - Enrofloxacine

FTA: Fast Technology for analysis of nucleic Acid

GALVmed: Global Alliance for Livestock Veterinary Medicine

GIS: Geographic Information System

HAPT1: High Affinity Pentamidine Transporter 1

HAT: Human African Trypanosomiasis

HCT: Haematocrit Centrifugation Technique

IFAT: Indirect Fluorescent Antibody Test

ISM: Isometamidium Chloride

ITM: Institute of Tropical Medicine

ITS-1 rDNA: Internal Transcribed Spacer 1 ribosomal DNA

ITS-1 TD PCR: Internal Transcribed Spacer 1 "Touch Down" Polymerase Chain Reaction

LACOMEV: Laboratoire de Contrôle des Médicaments Vétérinaires

LAPT: Low Affinity Pentamidine Transporter

MA: Marketing Authorization

m-AECT: miniature-Anion Exchange Centrifugation Technique

MatE: Multi Antimicrobial Extrusion Family

MDA: Multiple Displacement Amplification

MEP: Mitochondrial Electrical Potential

MFS: Major Facilitator Superfamily

MPS: Mononuclear Phagocytic System

Ngo's: Non-Governmental Organization

OTC: Oxytetracycline

PAGE: Poly Acrylamide Gel Electrophoresis

PATTEC: Pan African Tsetse and Trypanosomiasis Eradication Campaign

PCR: Polymerase Chain Reaction

PCV: Packed Cell Volume

RESCAO: Réseau d'Epidemio-Surveillance de la Chimiorésistance aux trypanocides et acaricides en Afrique de l'Ouest

RFLP: Restriction Fragment Length Polymorphism

rpm: Rotation Per Minute

RS: Reference Sample

SSCP: Single Strand Conformation Polymorphism

Ssu-rDNA: Small Sub-Unit Ribosomal DNA

T. brucei: *Trypanosoma brucei*

T. congolense: *Trypanosoma congolense*

T. vivax: *Trypanosoma vivax*

TbAT1: *Trypanosoma brucei* Adenosine Transporter 1

TBE: Tris Borate EDTA

TC: Tetracycline

TcoAT1: *Trypanosoma congolense* Adenosine Transporter 1

TcoNT10: *Trypanosoma congolense* Nucleoside Transporter 10

TDR: Trypanocidal Drug Resistance

TRYRAC: Trypanosomosis Rational Chemotherapy

UEMOA: Union Economique et Monétaire Ouest-Africaine

VAT: Variable Antigen Type

VR: Variable Region

VSG: Variant Surface Glycoprotein

WECATIC: Integrated Control of Ticks and Tick-borne diseases emerging in West and Central Africa

WHO: World Health Organization

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General Introduction

African trypanosomosis are debilitating and even deadly diseases in humans as well as in animals. Most species of *Trypanosoma* are transmitted by tsetse flies present in Africa only. Therefore, the spread of the disease follows the distribution of the vector, except for *Trypanosoma vivax* (*T. vivax*), which can be transmitted mechanically, and covers an area of approximately 9 million of Km^2, a third of the African continent (Budd, 1999).

Human African Trypanosomosis (HAT), better known as sleeping sickness, occurs in 36 sub-Saharan African countries with *Trypanosoma brucei gambiense* accounting for more than 98% of the reported cases (Simarro et al., 2011; WHO, 2013). At the end of the nineties, approximately 30,000 people were suffering of sleeping sickness. After continued control efforts, the number of new cases reported to the World Health Organization (WHO) dropped significantly from 9878 in 2009 to 7197 cases in 2012 (WHO, 2013). While sleeping sickness is clustered in specific foci, Animal African Trypanosomosis (AAT) is widely distributed with about 50 million head of cattle and other livestock species that are at risk of the disease (Mattioli et al., 2004). Thus, AAT represents a major constraint for livestock breeding in sub-Saharan Africa, and the annual direct and indirect losses due to AAT are estimated at 4.5 billion USD (Affognon, 2007). Indeed, in tsetse infected areas, it is estimated that half of the human population suffers from food insecurity and that 85% of the poor living in rural areas depend on agriculture for their livelihood (Mattioli et al., 2004). Considering the serious socio-economic impacts of AAT on poor rural populations, the conference of the presidents of the Organization of African Union held, in Lomé (Togo) in 2000, decided the creation of the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC). The main objective of this program is to improve food security and therefore reduce poverty by the eradication of tsetse flies.

However, eliminating tsetse flies from 9 million of km^2 of the African continent is a highly ambitious, costly and laborious project (Budd, 1999). Likewise, an alternative method such as a vaccine development is unlikely to be effective in the near future (Kinabo, 1993; Magez et al., 2010; Magez and Radwanska, 2009). Therefore, the challenge remains to make an optimal use of the few old trypanocidal compounds existing until the development of new drugs. Indeed, the control of livestock trypanosomosis in West Africa currently relies on three main molecules i.e. isometamidium chloride (ISM), homidium (bromide or chloride) and diminazene

aceturate (DA), which represent respectively 40%, 26% and 33% of the total trypanocidal drug market by value (Sones, 2001).

Moreover, the privatization and the liberalization of veterinary services that started since the 90s in West Africa (Coulibaly, 2004) have led to a situation in which drug administration is often in the hands of cattle farmers or extension workers, who may be un- or under-skilled in AAT differential diagnosis (Van den Bossche et al., 2000) and/or with different levels of know-how (Grace et al., 2009). These factors contribute to the spread of trypanocidal drug resistance (TDR) in the cotton belt of West Africa (Clausen et al., 2010; Talaki, 2008; Talaki et al., 2006) and beyond in 17 African countries (Delespaux et al., 2008). In the context of this thesis, TDR is defined as the decrease or absence of sensitivity of the trypanosome strains to standard quality trypanocidal drugs at the dose recommended by the manufacturer and administered according to the good veterinary practices. This is the case when the parasite is or adapted itself genetically to survive the toxic effects of the drug. This TDR is a quantitative trait varying between a slight decrease of sensitivity to a complete resistance against the toxic effects of the drug. Treatment failure is used when an animal was treated and is not cured, whatever the reason of the failure (wrong dilution, abscess formation, sub quality of the drug, genetically resistant parasite, etc.). While the costs of inappropriate drug use and lost production can be met largely by the farmer who misuses the drug, the costs of drug resistance are met by the entire society and the future generations. Therefore, our study aims to have a better insight on the problem of TDR in West Africa using molecular tools and to propose alternative methods for the management of this phenomenon.

Reference List

Affognon, H., 2007. Economic analysis of trypanocide use in villages under risk of drug resistance in West Africa. PhD thesis. In. Gottfried Wilhelm Leibniz University of Hannover, Germany. 195p.

Budd, L., 1999. DFID-funded tsetse and trypanosome research and development since 1980. Vol. 2. Economic analysis. DFID Livestock Production, Animal Health and Natural Resources Systems Research Programmes, Aylesford, UK. 123p.

Clausen, P.H., Bauer, B., Zessin, K.H., Diall, O., Bocoum, Z., Sidibe, I., Affognon, H., Waibel, H., Grace, D., Randolph, T., 2010. Preventing and containing trypanocide resistance in the cotton zone of West Africa. Transbound. Emerg. Dis. 57, 28-32.

Coulibaly, H., 2004. Organisation des services vétérinaires dans les pays en développement d'Afrique de l'Ouest. Rev. sci. tech. Off. int. Epiz. 23, 361-373.

Delespaux, V., Geysen, D., Van den Bossche, P., Geerts, S., 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol. 24, 236-242.

Grace, D., Randolph, T., Affognon, H., Dramane, D., Diall, O., Clausen, P.H., 2009. Characterisation and validation of farmers' knowledge and practice of cattle trypanosomosis management in the cotton zone of West Africa. Acta Trop. 111, 137-143.

Kinabo, L.D., 1993. Pharmacology of existing drugs for animal trypanosomiasis. Acta Trop. 54, 169-183.

Magez, S., Caljon, G., Tran, T., Stijlemans, B., Radwanska, M., 2010. Current status of vaccination against African trypanosomiasis. Parasitology 137, 2017-2027.

Magez, S., Radwanska, M., 2009. African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. Future Microbiology 4, 1075-1087.

Mattioli, R.C., Feldmann, G., Hendrickx, W., Wint, J., Jannin, J., Slingenbergh, J., 2004. Tsetse and trypanosomiasis intervention policies supporting sustainable animal-agricultural development. Food, Agr. Environ. Food Agr Environ 2, 310-314.

Simarro, P.P., Diarra, A., Ruiz Postigo, J.A., Franco, J.R., Jannin, J.G., 2011. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. PLoS Negl. Trop. Dis. 5, e1007.

Sones, K., 2001. Pharmaceutical companies: partners or enemies ? ICPTV Newsletter 3, 19-21.

Talaki, E., 2008. Etude de la résistance des trypanosomes à l'isométamidium et au diminazène dans la zone cotonnière de l'Afrique de l'Ouest (Mali - Guinée - Burkina Faso). PhD thesis. In. Université Polytechnique de Bobo-Dioulasso / Institut de Développement Rural / Département d'Elevage, pp. 1-160.

Talaki, E., Sidibe, I., Diall, O., Grace, D., Barry, A.M., Djiteye, A., Bocoum, Z., Clausen, P.H., Randolph, T., Affognon, H., Hendrickx, G., Pangui, L.J., Belem, A.M., 2006. Répartition spatiale des trypanosomoses animales en relation avec la chimiorésistance dans la zone cotonnière de l'Afrique de l'Ouest (Mali et Guinée). Revue Africaine de Santé et de Productions Animales 4, 45-50.

Van den Bossche, P., Doran, M., Connor, R.J., 2000. An analysis of trypanocidal drug use in the Eastern Province of Zambia. Acta Trop. 75, 247-258.

WHO, 2013. Trypanosomiasis, Human African (sleeping sickness). In. http://www.who.int/mediacentre/factsheets/fs259/en/index.html

Chapter 1

Animal trypanosomosis and chemotherapy: a literature review

1.1. Introduction

In the "Union Economique et Monétaire Ouest Africaine" (UEMOA) countries (i.e. Benin, Burkina Faso, Côte d'Ivoire, Mali, Niger, Senegal, Togo, Guinea Bissau), agriculture contributes to 30% of the Gross Domestic Product and employs 50% of the active population (Renard et al., 2004). Livestock breeding contributes to 5% (Côte d'Ivoire) to 44% (Mali) of the agricultural Gross Domestic Product, with an average close to 40% for the Sahelian countries (Kamuanga et al., 2008). The part of livestock breeding in the Gross Domestic Product would be even more important in West Africa (up to 50%) when including the value of animal traction and manure in mixed crop-livestock systems widely represented in the region (Smith et al., 1996; Winrock International, 1992). Unfortunately, the development and intensification of livestock breeding in this African region is hampered among others by cross-border epizootic diseases such as AAT. As an introduction to the following chapters, we will provide some background information on (i) the parasites responsible for the disease especially *Trypanosoma congolense* (*T. congolense*) *and Trypanosoma vivax* (*T. vivax*) on which the thesis is focused, and on (ii) the disease: its epidemiology, pathogenesis, symptoms, diagnosis as well as the different strategies to control it in the presence or absence of TDR.

1.2. The trypanosomes

Trypanosomes are flagellated protozoan parasites that live in the blood, plasma, lymph and several tissues of their vertebrate hosts (Stephen, 1986). In West Africa, livestock are predominantly infected by two main species of trypanosomes: *T. vivax* and *T. congolense* (Kalu et al., 2001; Nakayima et al., 2012).

1.2.1. Taxonomy, morphology and structure

Trypanosomes belong to the phylum *Sarcomastigophora*, the order *Kinetoplastida,* the family *Trypanosomatidae* and the genus *Trypanosoma.* The genus *Trypanosoma* is divided into two groups (Hoare, 1972):

(i) Stercoraria (subgenera *Schizotrypanum, Megatrypanum* and *Herpetosoma*) in which

trypanosomes are produced in the hindgut of the vector and transmitted to the hosts by contaminating excrements.

(ii) Salivaria (subgenera *Duttonella, Nannomonas, Trypanozoon*): here the transmission occurs by inoculation when the vector injects its saliva at the time of the bite, which precedes the blood meal. Salivarian species are the only trypanosomes to exhibit antigenic variation (Stevens and Brisse, 2004), which allows the parasite to escape the host immune reaction. Apart from *T. theileri* belonging to the Stercoraria group, African trypanosomes belong to the Salivaria group.

Morphologically, *T. congolense* (subgenus *Nannomonas*) is the smallest of the pathogenic African trypanosomes, with an average size varying between 12 to 17 µm. The classic blood forms lack a free flagellum with a poorly developed undulating membrane. However, certain strains present a short flagellum and a more developed undulating membrane (Chartier et al., 2000). In stained specimens of *T. congolense,* the cytoplasm appears diffuse, the nucleus is centrally placed and the kinetoplast terminal or subterminal (Uilenberg, 1998). The PCR-RFLP approach targeting the 18S small ribosomal subunit gene (Geysen et al., 2003) allowed the identification of the three *T. congolense* sub-species i.e. *T. congolense* savannah type, *T. congolense* forest type and *T. congolense* kilifi type.

T. vivax (subgenus *Duttonella*) is longer (18 to 31 µm) with a free flagellum and a large terminal or subterminal kinetoplast. The nucleus is centrally placed and the undulating membrane is discrete even if some blood forms present a more developed undulating membrane (Chartier et al., 2000).

As for all protozoan parasites, trypanosomes are unicellular cells constituted by a mass of cytoplasm limited by a plasma membrane and containing different organelles as well as a nucleus. Electron microscopy has enabled to better understand the structure of trypanosomes. Thus:

- The plasma membrane or periplasm is composed by a stratum of microtubular fibers and a membrane of 8 to 10 nm of thickness. The latter is constituted by 3 stratums (extern, median and intern) of which the extern stratum is sometimes covered by an

amorphous layer of 12 to 15 nm of thickness, secreted by the cell. This layer, composed of glycoproteins, is observed in blood forms of livestock trypanosomes and is named the surface antigen (Uilenberg, 1998). This surface antigen is responsible of the immune evasion of trypanosomes in their vertebrate hosts (see later in the pathogenesis paragraph).

- The flagellum starts with a small invagination of the plasma membrane called flagellar pocket and is prolonged by the blepharoplast, a transition zone and the flagellum itself. The flagellar pocket is the only site of exo- and endocytosis. Several flagellar pocket-associated proteins were detected to contribute to the trafficking of nutrients, to the recycling of the VSG and to the virulence (Field and Carrington, 2009).

Besides the usual organelles of protozoan cells such as the vacuolar apparatus (endoplasmic reticulum, Golgi apparatus, etc.), trypanosomes have a specific organelle called glycosome containing enzymes responsible for the metabolism of glucides. Another particularity of trypanosomes is a unique big mitochondrion called kinetoplast containing the mitochondrial DNA (Figure 1.1). The general organization of the genome (nuclear and mitochondrial) will be explained in the next paragraph.

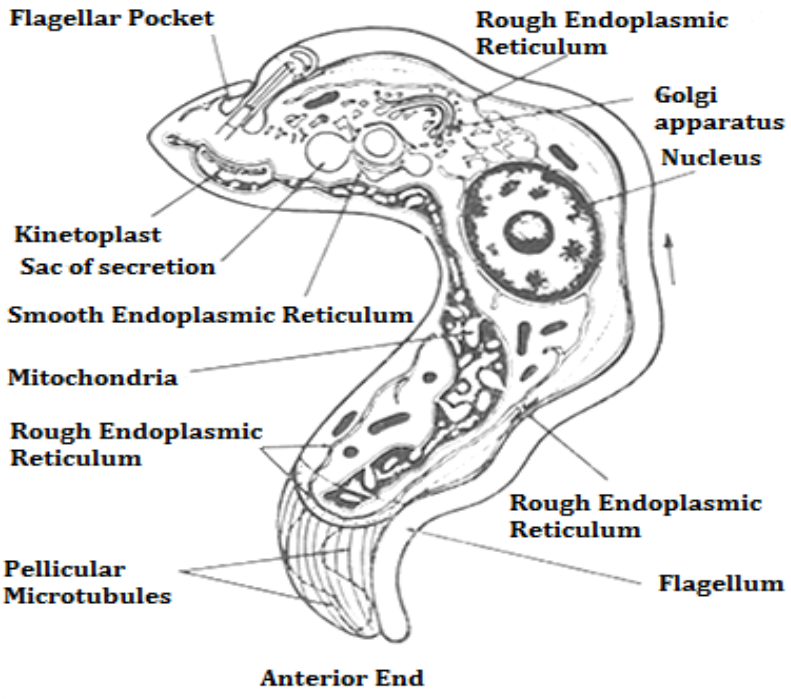

Figure 1.1: Ultrastructure of a bloodstream form of *T. congolense*
Source: http://ilri.org/infoserv/Webpub/fulldocs/Ilrad82/Trypano.htm

1.2.2. Genome

The nuclear genome shows inter- and intra-species plasticity in chromosome number and size, whereas the mitochondrial genome (Kinetoplast DNA or kDNA that accounts for 10-20% of the total cell DNA) varies in number of minicircles and within the variable region of maxicircles.

1.2.2.1. Nucleus

All trypanosome species contain a set of large chromosomes of which there are at least 11 pairs of chromosomes (I-XI) ranging from 1 Mb to more than 6 Mb (Melville et al., 1998; Melville et al., 2000). These megabase chromosomes represent about 80% of the nuclear genome and are called housekeeping chromosomes because they carry most of the genes involved in the basic functions of the trypanosome. Besides, there are several intermediate size chromosomes of 200 to 900 kb of uncertain ploidy, representing between 0.2 to 1% of the genome. In addition to megabase and intermediate chromosomes, approximately 100 linear mini-chromosomes of 50 to 150 kb are present accounting for about 20% of the nuclear DNA content and serving as repositories for variant surface glycoproteins (VSG) genes (El-Sayed et al., 2000).

1.2.2.2. Kinetoplast DNA

Trypanosomes are characterized by a kDNA network, which represents the mitochondrial genome that accounts for 10-20% of the total cell DNA (Hajduk et al., 1992). Structurally, the kDNA occurs within the cell as a flattened disc of intercalated DNA circles called maxicircles and minicircles.

1.2.2.2.1. Maxicircles

Basically, maxicircles are composed of two distinct regions: the variable region (VR) and the coding region of 3-6 kb and 17 kb, respectively. While the latter is generally well conserved, the VR varies in length and gene sequence content between species and isolates (Myler, 1993). There are approximately 50 maxicircles per cell; they encode for the 9S and 12S ribosomal sub-units and for enzymes implicated in the electron transport system (respiratory chain), which generates energy during the insect phase of the

parasite life cycle. Finally, maxicircles size varies between species of trypanosomes and is on average 20 times higher than that of the minicircles.

1.2.2.2.2. Minicircles

With a size of about 1 kb, minicircles are variable among species while they are constant within species. There are approximately 5000-10000 minicircles per cell, highly heterogeneous with up to 200 different sequence classes per cell (Shapiro and Englund, 1995). Structurally, minicircles consist of two regions: a small conserved region constant in all minicircles of each species and a large non-conserved region which varies between minicircles. Contrary to maxicircles, minicircles does not code for any proteins but they encode guide RNAs that play a central role in mRNA editing (Melville et al., 2004).

1.3. Vectors of African trypanosomes

With the exception of *T. equiperdum*, which is sexually transmitted, African trypanosomes are transmitted to the hosts by haematophagous insects. Tabanids and stable flies, rarely hippoboscids, are responsible for the mechanical transmission of *T. vivax* and *T. evansi* (Desquesnes and Dia, 2003). In these cases, the parasites do not multiply nor undergo developmental changes within their insect vector. However, most of African trypanosomes pathogenic to livestock are cyclically transmitted by tsetse flies. These obligate haematophagous flies belong to the order of the *Diptera,* family of *Glossinidae,* genus *Glossina.* This genus is divided in three subgenera: *fusca, morsitans* and *palpalis* (Buxton, 1955) in which about thirty species and subspecies can be recognized.

Tsetse flies are widely distributed across the African continent, mostly in open woody savannahs or dense forest galleries providing optimal conditions for their survival and reproduction (Figures 1.2 & 1.3).

All tsetse species

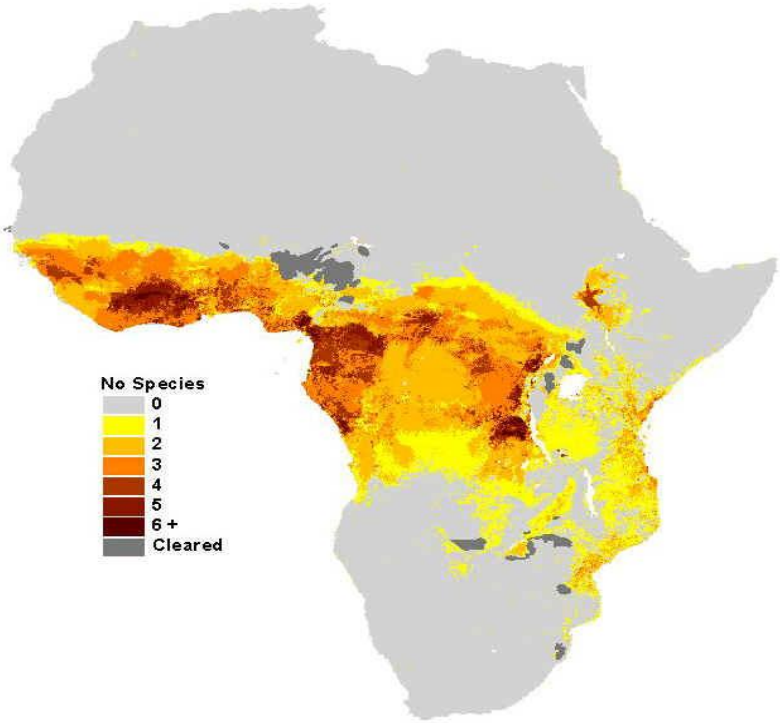

Figure 1.2: Distribution and number of tsetse fly species in Africa

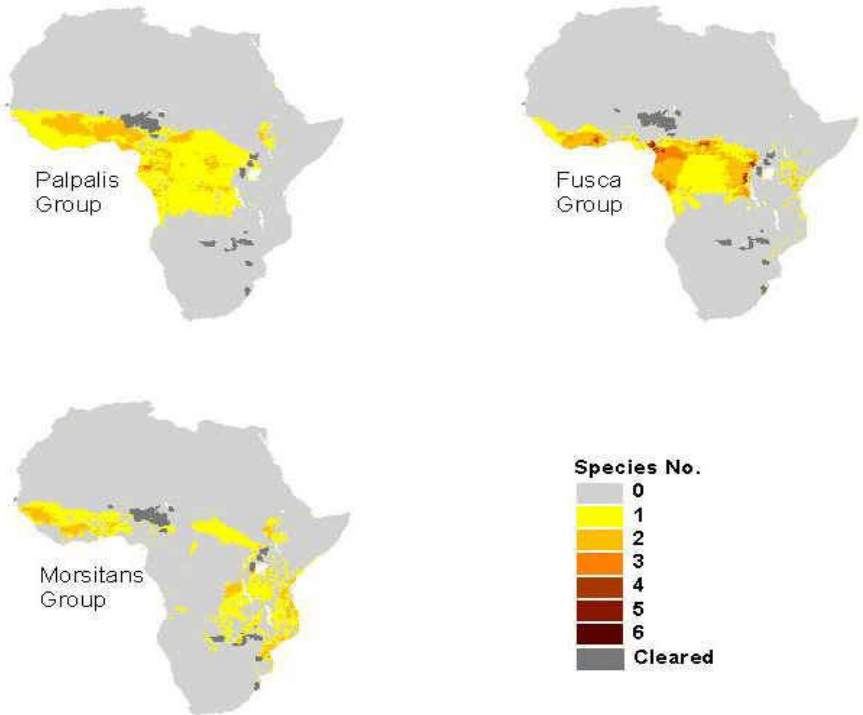

Figure 1.3: Distribution and number of tsetse fly species belonging to the *fusca, morsitans* and *palpalis* groups in Africa
Source of figures 1.2 and 1.3: http://ergodd.zoo.ox.ac.uk/livatl2/tsetse.htm

1.4. Life cycle

During their feeding on a trypanosome-infected animal, tsetse flies ingest the blood stream form trypanosomes that have to go through a number of morphological, biochemical and physiological transformations within the fly to become infectious (metacyclic forms) again for the mammalian host. For *T. vivax*, the entire cycle takes place in the proboscis whereas for *T. congolense,* the development occurs in the midgut and proboscis (see table 1.1). Once the final infectious metacyclic trypanosomes are present in the tsetse fly, they will be injected in the dermis of the mammalian host at each blood-feeding event (Figure 1.4).

Within the mammalian host, the trypanosomes transform, start replication and are transported to the draining lymph node through the afferent lymphatic system. Then through the efferent lymphatic system, parasites reach the thoracic lymph duct and finally enter the blood circulation where they continue to replicate (Akol and Murray, 1986). While *T. congolense* is preferentially localized in small blood vessels and capillaries, *T. vivax* is also seen in the tissues of their host. The duration of the pre-patent period generally ranges from 1 to 3 weeks depending on the species and strain of trypanosomes and on the immune status of the host (Clausen et al., 1993).

Table 1.1: Site of development in *Glossina* spp of the different *Trypanosoma* species (Hoare, 1970; Van Den Abbeele et al., 1999)

Subgenus	Species	Midgut	Proboscis	Salivary glands
Dutonella	*T. vivax*	No development	Trypomastigotes Epimastigotes Final infective stage: metacyclic trypomastigotes	No development
Nannomonas	*T. congolense* *T. simiae* *T. godfreyi*	Trypomastigotes	Trypomastigotes Epimastigotes Final stage: metacyclic trypomastigotes	No development
Trypanozoon	*T. b. brucei* *T. b. rhodesiense* *T. b. gambiense*	Trypomastigotes	Trypomastigotes and epimastigotes	Trypomastigotes and epimastigotes Final stage: metacyclic trypomastigotes

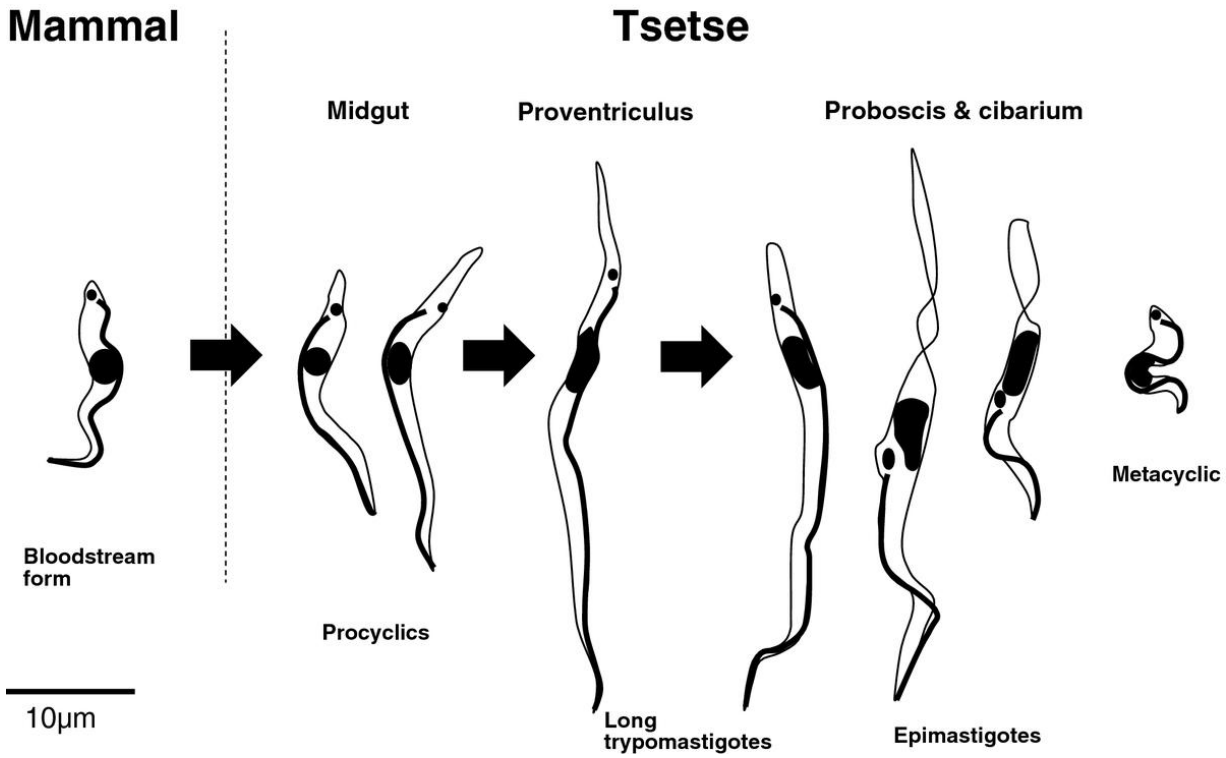

Figure 1.4: *Trypanosoma congolense* life cycle stages (Peacock et al., 2012)

1.5. Epidemiology

1.5.1. Some general aspects of the epidemiology of trypanosomosis in livestock

The epidemiology of AAT depends on three main factors: (i) the virulence of the parasite, (ii) the distribution of the vector and (iii) the response of the host (trypanosensitivity). These factors are modulated by local environmental changes that can substantially affect their dynamics and consequently influence the disease transmission patterns. Anthropogenic environmental changes, such as the increased human pressure and the simultaneous demand for arable land result in deforestation and loss of suitable habitats for tsetse flies and their hosts. In West Africa, over the past 150 years, the extents of forest areas have reduced to about 13% and currently few are in pristine condition (Larsen, 2008).

Considering the cyclical transmission of the parasite, the diversity of tsetse fly habitats considerably affects the interactions among vectors, hosts (livestock and/or wildlife) and parasites (levels of virulence) (Van den Bossche et al., 2010). For the savannah species (*G. morsitans* sp.), the disease is widespread due to the large dispersion of these

flies. For the riverine species (*G. palpalis gambiensis, G. tachinoides*), the areas of contacts with the hosts are limited. In this case, the risk of infection does not only depend on the density of the flies but also on the intensity of space-time interfaces between tsetse and livestock (de La Rocque et al., 2001). Tsetse flies are living for millions of years in proximity of wildlife constituting the sylvatic trypanosomosis transmission cycle. Nowadays, the importance of livestock as a source of food for tsetse flies is increasing as a consequence of human encroachment (deforestation for cultivation) and the reduction in wildlife species (Van den Bossche et al., 2010). These drastic changes in tsetse flies habitats and hosts availability have resulted either in their elimination or in many cases in their adaptations to those new conditions (Bourn et al., 2001). The use of specific microclimatic niches (Terblanche et al., 2008; Van den Bossche et al., 2010) and their opportunistic feeding behavior regarding the choice of the host made this adaptation possible (Krafsur, 2009).

Parasite virulence is also an important factor influencing the epidemiology of AAT. For example, West Africa's *T. vivax* strains are rapidly fatal when compared to those of East and Central Africa. Similarly, *T. congolense* induces a more chronic disease in West Africa compared to East and Central Africa except in the vicinity of protected game reserves (Leak, 1999; Van den Bossche and Delespaux, 2011). Moreover, within the *T. congolense* group, the three subgroups i.e. Savannah, Kilifi and Forest (Hide and Tait, 2004) show important differences in virulence with the Savannah subgroup strains being the most virulent (Bengaly et al., 2002a; Bengaly et al., 2002b). Even within *T. congolense* Savannah subgroup, substantial differences in virulence are observed between strains (Masumu et al., 2006) and between transmission cycles, with significantly higher virulent strains in the sylvatic transmission cycle (Van den Bossche et al., 2011). These differences in virulence associated with the level of tolerance of the livestock determine the level of endemicity of the disease in a particular area.

Actually, the response of the host to the infection depends on its innate susceptibility, modulated by the level of activity of its immune system (food availability, co-infections, etc…). In West Africa, Zebu breed are the most trypanosensitive cattle whereas Sanga and Shorthorn breeds are more trypanotolerant. In the vicinity of game reserves, the fact that wild animals are the reservoirs of the disease makes the epidemiology of AAT even more complex. Indeed, the higher virulent *T. congolense* strains of the Savannah

subgroup observed in wild animals may explain the rapidly fatal issue of trypanosomosis in those settings (Van den Bossche et al., 2011).

1.5.2. Livestock management practices

In sub-Saharan Africa, livestock management practices influence the epidemiology of AAT. Three main systems are co-existing i.e. (i) transhumance (nomadic breeders), (ii) sedentary agro-pastoralists and (iii) ranching (Cuisance et al., 2003).

The alternation of seasons imposes to nomadic livestock breeders a migration for reaching areas suitable for grazing and watering. During the dry season, the dispersion of savannah flies is reduced and the risk of contracting the disease is sufficiently limited to allow Fulani, the best-known West Africa's nomadic cattle keepers, to migrate with their livestock from North to South, far from tsetse fly areas (Affognon et al., 2009; Courtin et al., 2010). However, these animals will be in contact with riverine tsetse flies populations at watering points in gallery forest along the rivers (Figure 1.5). In the rainy season, cattle grazing in the Sahel or in Northern Sudanese savannah do not encounter tsetse flies. Nevertheless, animals are harassed by other haematophagous flies, which can maintain *T. vivax* – trypanosomosis within the herd (Chartier et al., 2000; Cuisance et al., 2003).

The sedentary cattle grazing in the South-Sudanese savannah during the rainy season are in contact with the species belonging to the *morsitans* group (*G. longipalpis, G. morsitans submorsitans,* etc*…).* During the dry season, the sedentary cattle are in contact with the riverine flies (*G. palpalis, G. tachinoides*, etc) when they penetrate into the forest gallery. In the Guinean zone, these cattle are confronted year round with both the savannah and forest/riverine flies (Leak, 1999). Finally, in commercial 'farms' animals are usually treated when clinical signs are seen. The cattle are then in good condition as long as the efficacy of the drugs is preserved.

Figure 1.5: Zebu cattle on a watering point at the Mouhoun river (Burkina Faso)

1.6. Pathogenesis

Initial replication of metacyclic trypanosomes begins at the site of inoculation, inducing a swelling and a sore called 'chancre'. This 'chancre' disappears after a few days (3 to 15 days) when the trypanosomes spread to the lymph nodes and blood and continue to replicate (Chartier et al., 2000). After an infection has become established, the B-lymphocytes of the mammalian host produce anti-VSG antibodies (IgG) that lyse a large number of the trypanosomes and result in the development of immunes complexes. These are removed by phagocytes and presented to lymphoid cells. The attachment of antigen-antibody complexes to red blood cell membranes contribute to the damage and lysis of the erythrocytes. Moreover, the lysis of the trypanosomes release many harmful substances in the extracellular environment, such as haemolysins and enzymes (proteases, phospholipases and neuraminidases, etc), which directly damage the host's erythrocytes membranes. The elimination of large numbers of damaged erythrocytes from the blood circulation occurs soon after the beginning of the parasitaemia, by cells of the mononuclear phagocytic system (MPS), and produces a decrease in the Packed Cell Volume (PCV). Some trypanotolerant cattle (e.g. Baoule) control the parasitaemia quite effectively (earlier and higher antibody responses to the first peak of

parasitaemia) when compared with similarly infected Zebu cattle (Connor and Van den Bossche, 2004). The PCV values of infected trypanotolerant animals are definitely higher than that of susceptible ones when they are well maintained (deworming, food in adequate quantity, absence of concomitant infection, etc).

Anaemia is then largely attributable to an increased rate of erythrophagocytosis in the early phase of infection. Nevertheless, there is no single cause of the anemia in AAT. Indeed, as a response to the trypanosomal infections both IgM and IgG antibodies are produced. While the IgM appear to be directed mainly against VSG antigens, IgGs are oriented against both the somatic trypanosomal antigens and the host's own cells (Connor and Van den Bossche, 2004). Anti-erythrocyte antibodies contribute to the anemia of AAT and it is likely that a similar mechanism contributes to leukopenia.

However, anti-VSG antibodies do not clear the infection as the trypanosome produces many different surface-coat glycoproteins (10^7 Variable Antigen Types or VAT) and change their surface glycoprotein to evade the host immune response (Baral, 2010). Thus, there is a persistent fluctuating parasitaemia that results in continuing cycles of trypanosome replications, antibody production, immune complex development, and changing of surface-coat glycoproteins. Furthermore, one of the most significant and complicating factors in the pathogenesis of AAT is the immunosuppression that occurs following infection by these parasites. This marked immunosuppression lowers the host's resistance to other infections and thus results in secondary diseases, which greatly complicate both the clinical and pathological feature of AAT. It has been suggested that immunosuppression is mediated by both the macrophages and the T cells (Tabel et al., 2008). As a result of this, antigenic variation and immunosuppression, conventional vaccination strategies against AAT are not effective (Baral, 2010; Magez et al., 2010). Recently, alternatives approaches were explored based on camelid antibodies (e.g. use of Nanobodies®) (Magez et al., 2010; Magez and Radwanska, 2009). Promising results were obtained using low molecular weight VSG-specific trypanolytic nanobodies that impede endocytosis (Stijlemans et al., 2011). Further research is necessary in order to know if this technique developed with *T. brucei* as model could be adapted to *T. congolense* or *T. vivax*.

Significant tissue lesions are observed in AAT and depend on the species of trypanosome. Considered to be mainly intravascular parasites, *T. congolense* and to a lesser extent *T. vivax*, cause changes in the endothelium of capillaries, and thus indirectly provoke damage to adjacent tissues. The severity of endothelial damages also depends on the host-parasite interaction. Indeed, in *T. congolense* infections a generalized dilatation of capillary beds is observed, which alters the haemodynamics. In contrast, *T. vivax* infections commonly cause disseminated intravascular coagulation (Connor and Van den Bossche, 2004).

1.7. Clinical signs

Due to the fact that simultaneous infections with more than one trypanosome species and/or with other haemoparasites (*Babesia* spp., *Theileria* spp., *Anaplasma* spp. and *Ehrlichia* spp.) are very common (Nyeko et al., 1990), it is difficult to attribute the clinical signs to a given parasite. Moreover, the disease may have acute, chronic or sub-clinical forms complicating its recognition. Nonetheless, the major clinical sign observed in AAT is anemia, followed invariably by an intermittent fever, weight loss, roughness of the hair coat and whimpering (Taylor and Authié, 2004). The severity of the clinical response depends on the species and the breed of the affected animal but also on the trypanosome species (Table 1.2), the size of the inoculum and the virulence of the infecting trypanosomes. For example, *T. brucei brucei* is very well tolerated by cattle but is rapidly life threatening for horses and dogs (Allsopp et al., 2004). Even within a same species virulence might vary a lot as is the case for *T. congolense* (Masumu et al., 2006).

Table 1.2: Susceptibility of livestock species to the pathogenic trypanosome species. Adapted from (Soltys, 1963)

Livestock Species	Trypanosome species						
	T. congolense	*T. simiae*	*T. vivax*	*T. brucei*	*T. evansi*	*T. equiperdum*	*T. suis*
Cattle	+++	±	++	+	+	–	–
Sheep	++	+	++	+++	++	–	–
Goat	++	+	++	+++	++	–	–
Pig	+	+++	–	+	–	–	+++
Horse	++	–	++	+++	++	+	–
Camel	++	–	–	++	++	–	–

+++: very susceptible; ++: susceptible; +: less susceptible; –: no infection

1.8. Diagnosis

1.8.1. Clinical diagnosis

Due to the absence of any pathognomonic clinical sign for AAT, the clinical diagnosis is somewhat difficult. Nonetheless, anemia, pyrexia, weight loss, roughness of the hair coat, hypertrophy of peripheral lymph nodes, abortion, reduced milk yield and, in absence of treatment, death are often observed in cattle affected by the acute form of the disease (Eisler et al., 2004). Whatever the clinical forms of evolution of AAT (acute, subacute and chronic forms), it is essential to confirm the presence of the parasite in blood or lymph node smears by using the following parasitological tests.

1.8.2. Parasitological diagnosis

1.8.2.1. Direct microscopic examination

The examination of a drop of fresh blood between slide and coverslip can be of great use in the field to demonstrate the parasitaemia of animals under observation or treatment or to determine the health status of a herd throughout the seasons. Using a light microscope at 400x magnification, the species of trypanosomes can be determined by examining their size and movement patterns. *T. congolense* appears short (size of about 8 to 24µm) with a poorly developed undulating membrane; the free flagellum is absent or very brief and the trypanosome is stuck on erythrocytes and moves slowly. In contrast, *T. vivax* quickly crosses the field of the microscope; it is a larger trypanosome measuring about 18 to 31µm with a free flagellum and a less developed undulating membrane. *T. brucei* has a well-developed undulating membrane and moves freely but slower than *T. vivax* and often describes little circles. Microscopic examination of fresh blood films is simple and inexpensive but lacks sensitivity, with a detection limit of about 10^4 trypanosomes /ml of blood (Uilenberg, 1998).

Thin or thick blood smears fixed in methanol or acetone and colored with May-Grünwald-Giemsa as well as stained lymph node smears (Uilenberg, 1998) are mostly used for accurate trypanosome identification and are found to be more sensitive than the fresh blood film (Kalu et al., 1986; Paris et al., 1982). However, none of these techniques is sensitive enough to detect the low parasitaemia usually observed in the field when compared to concentration techniques.

1.8.2.2. Concentration techniques

The Haematocrit Centrifugation Technique (HCT) is based on the centrifugation of microhaematocrit capillary tubes containing the blood sample. Afterwards, the buffy coat/plasma junction is observed under a microscope at 250x magnification in a special Woo chamber, allowing the suppression of the refraction of the light on the capillary tube (Woo, 1970). The Buffy Coat Technique (BCT) developed by Murray et al. (1977) is a variant of the HCT. The capillary tubes are cut at the level of the buffy coat/plasma interface. The buffy coat is extruded on a slide, covered with a coverslip and examined under the microscope at 400x magnification with dark ground or phase contrast illumination. In addition to be more sensitive, these two techniques have also the advantage of measuring the Packed Cell Volume (PCV) or haematocrit.

Another available method that is not commonly used for animals in field conditions is the miniature-Anion Exchange Centrifugation Technique (m-AECT). This technique using miniature anion-exchange columns for the separation of trypanosomes from erythrocytes prior to concentration by centrifugation (Lumsden et al., 1979), has recently been improved for sleeping sickness diagnosis and staging (Büscher et al., 2009). Due to the different electric charges of the trypanosomes and the erythrocyte surface, the erythrocytes get captured in the column whereas the trypanosomes are eluted.

1.8.3. Serological diagnosis

Several antibody-detecting tests are used for the diagnosis of trypanosomal infection. These tests only confirm the contact between the host and the parasite without specifying whether the infection is still active or has been cleared (Uilenberg, 1998). The most commonly used tests in cattle are the Indirect Fluorescent Antibody Test (IFAT), the antibody Enzyme-Linked Immunosorbent Assay (Ab-ELISA) and the Card Agglutination Test for *T. evansi* (CATT/*T. evansi*).

In the IFAT, the antigen is constituted of a blood smear containing fixed trypanosomes. The primary Abs are detected by commercially available secondary antibodies conjugated with a fluorophore (like the fluorescein isothiocyanate or FITC). Despite the fact that antigen production is easy, this test has the disadvantage of not being

sufficiently species specific. Cross reactivity between species are frequently observed. Moreover, the commercial conjugates are expensive and only few numbers of sera can be examined in a given period as the method is rather labor intensive (Uilenberg, 1998).

The principle of the Ab-ELISA is quite similar to IFAT. However, when compared to IFAT, the indirect ELISA proved to be more sensitive (Eisler et al., 2004). The test is more useful for epidemiological purposes than for individual routine diagnosis, and in areas with a low AAT prevalence. When the fly challenge is high the method is of limited interest as serological prevalence can then reach 80 – 90% (Van den Bossche et al., 1999; Van den Bossche et al., 2000b). The prevalence of anti-trypanosomal antibodies is a sensitive indicator of the impact of tsetse control operations on disease challenge and for confirming the disease-free status of animals in areas from which tsetse has been eliminated (Van den Bossche et al., 2000a). Serology can then be performed on the new born calves which should not have been in contact with trypanosomes if the eradication was successful. Nevertheless, Ab-ELISA is quite expensive and the antigens are more difficult to produce than for IFAT (Connor and Van den Bossche, 2004).

The CATT/*T. evansi* test is a direct card agglutination test for the detection of antibodies to 'surra' i.e. *T. evansi* infection in blood, serum or plasma (Desquesnes et al., 2013) of various animals species such as, camels (Ngaira et al., 2003; Njiru et al., 2004) and water buffaloes (Davison et al., 2000; Hilali et al., 2004). It consists of mixing a drop of whole blood or serum on a plastic card with fixed and stained trypanosomes as antigen, and the test is positive when the antigen agglutinates. This test is easy to carry out in the field, although its specificity and sensitivity need to be enhanced (Uilenberg, 1998). More recently, a highly specific and sensitive ELISA (ELISA/rrISG75) using a recombinant non-variable antigen was developed for diagnosis of 'surra' in camels (Tran et al., 2009).

Efforts were made at the International Laboratory for Research on Animal Diseases (ILRAD) in Nairobi (Kenya), to develop tests based on the detection of circulating antigens (Nantulya et al., 1987). This test is based on the use of monoclonal antibodies (Mabs) against invariable antigens. Antigen-detecting ELISA (Ag-ELISA) would have allowed the detection of circulating trypanosomal antigens and therefore confirm the occurrence of an active infection (Eisler et al., 2004). However, in general this test

appears to be less sensitive, particularly for the detection of *T. vivax* infection (Desquesnes and de La Rocque, 1995) and even false positive results and cross-reactions occurs frequently with the Ag-ELISA for the *T. congolense* and *T. brucei* groups (Uilenberg, 1998). The lack of reproducibility and specificity of the Ag-ELISA has led to the abandon of it (Desquesnes and de La Rocque, 1995).

1.8.4. PCR-based methods

PCR is based on the use of an enzyme, the DNA polymerase, in presence of nucleotides in a buffered environment, which amplifies a specific target sequence of DNA, until sufficient material is produced to be detected. Generally, once the specificity of the primers has been established, the size of the amplicon is sufficiently characteristic for diagnostic purposes (Desquesnes and Davila, 2002).

The advantages of the classical PCR include: the easier and longer conservation of the samples i.e. buffy coats collected on Whatman filter paper N°4 or Whatman FTA cards (10 years shelf life at room temperature) and the higher specificity and sensitivity compared to the other parasitological techniques (20 to 500 times more sensitive). Indeed, PCR can detect between 1 to 20 trypanosomes per ml. However, some drawbacks have been reported such as *(1)* blood containing inhibitory substances (e.g. haemoglobin) that can lead to false negative results; *(2)* extreme care is necessary to avoid DNA contamination, especially when considering the mode of conservation of the samples on filter papers and *(3)* for each trypanosome species a separate PCR is necessary using specific primers, except when using the Internal Transcribed Spacer 1 ribosomal DNA (ITS-1 rDNA) as PCR target in a multiplex PCR (Desquesnes et al., 2001).

Alternatively, PCR associated with Restriction Fragment Length Polymorphism (PCR-RFLP), allows for the identification of different trypanosome (sub)-species in one test i.e. a “pan-trypanosome” test for all bovine trypanosomes (Geysen et al., 2003). Indeed, PCR-RFLP targeting the 18S ribosomal small sub-unit DNA (Ssu-rDNA) has a higher sensitivity than multiplex PCR using ITS-1 primers. However, the newly developed ITS-1 “Touch Down” PCR (ITS-1 TD PCR) in addition to achieve a comparable sensitivity, is less-time consuming (a single amplification step and no enzymatic restriction are needed) and cheaper compared to the 18S-PCR-RFLP technique (Tran et al., pers. Comm.).

1.9. Control of Animal African Trypanosomosis

The control of AAT includes the use of trypanotolerant cattle, vector control and the use of trypanocidal drugs or any combination of the methods according to the specific conditions of an area. The choice of a strategy will depend mainly on the tsetse fly challenge, the susceptibility of the host, the presence/absence and type of drug resistance (simple or multiple) and the breeding system (transhumant, sedentary not confined, sedentary confined, …).

1.9.1. Use of trypanotolerant cattle

Breeding trypanotolerant cattle (*Bos taurus*) is an interesting alternative for the control of AAT. When properly housed and fed, these cattle can maintain good productivity levels in tsetse infested areas. Trypanotolerant cattle can be divided into two groups: (i) the short-horned cattle represented by the breeds *Baoulé*, *Sumba*, *Muturu* of savannah, *Lagune*, bred in Côte d'Ivoire, Benin, Togo, Ghana, Nigeria, Burkina Faso and Northern Cameroon and (ii) the long-horned cattle living in Southern Senegal, Mali, northern Côte d'Ivoire, Guinea, Gambia, Liberia, Sierra Leone, Bissau Guinea, Burkina Faso. Long-horned cattle are represented by the breed *Ndama.* Due to their small size, farmers are somewhat reluctant to breed them (Uilenberg, 1998). This has led farmers to cross trypanotolerant livestock with Zebu to increase the size of the animals and the milk yield. The obtained crossbreds are partially trypanotolerant and represented by the breeds *Borgou* in Benin and Togo, *Méré* in Guinea, Burkina Faso, Côte d'Ivoire, *Bambara* in Mali and *Djakoré* in Senegal and Gambia.

1.9.2. Vector control

Fly populations can be down-regulated by chemical control including the use of insecticides by ground application, aerial spraying, and impregnation of traps / screens or spraying / pour-on application on the back line of host animals, spraying the belly and legs of cattle. This last method is cheap and easy to implement and take advantage of the specific tropism of heamatophagous flies for the lower parts of the animals (Bouyer et al., 2009; Bouyer et al., 2007). When the tsetse fly population is decreased by 95% or more in isolated areas that cannot be reinvaded, eradication can be achieved by the Sterile Insect Technique (release of sterile males). This technique is based on the fact that female flies only accept one single mating. If this mating is performed by a sterile

male, this female will never produce any offspring. Methods such as deforestation and elimination of the game animals that were used in early eradication campaigns, have been abandoned because of obvious environmental and ethical reasons (Uilenberg, 1998). Paradoxically, encroachment has the same effect and large areas are in this way freed from the burden of tsetse flies allowing agriculture and cattle breeding. However, livestock keepers often lead their animals to less degraded environments where grass and trees are still available and tsetse flies as well.

1.9.3. Trypanocidal drugs

Currently, in West Africa, three main molecules are used to deal with AAT: diminazene aceturate (DA), isometamidium chloride (ISM) and homidium salts (bromide or chloride).

1.9.3.1. Diminazene aceturate

Structurally, DA is an aromatic diamidine derived from Surfen (Jensch, 1958). The compound, marketed as aceturate salts, consists of two amidinophenyl linked by a triazene bridge: p,p-diamidinodiazoaminobenzene diaceturate tetrahydrate; N-1,3-diamidinophenyltriazene diaceturate tetrahydrate ($C_{22}H_{29}N_9O_6.4H_2O$, mol. wt. = 587,6; mol. wt. base = 281,2) (Figure 1.6) (Peregrine and Mamman, 1993).

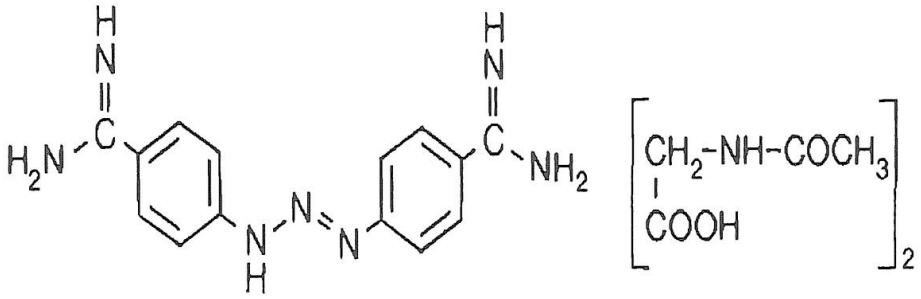

Figure 1.6: Molecular structure of diminazene aceturate (Peregrine and Mamman, 1993).

When taken up by the trypanosome, DA is mainly accumulated in the kDNA where it binds through specific interactions with sites rich in adenine-thymine (A-T) base pairs.

The compound has a higher affinity to 5'-AATT-3' than to 5'-TTAA-3' regions of DNA (Peregrine and Mamman, 1993). It has been demonstrated that DA binds to the double-stranded DNA via the minor groove through electrostatic and hydrogen-bond forces. Therefore, DA interferes with the synthesis of RNA primers, resulting in the accumulation of replicating intermediates and subsequent inhibition of kDNA replication. Moreover, Shapiro and Englund (1990) have shown that DA inhibits the mitochondrial type II topoisomerases in trypanosomes which blocks the DNA replication.

The drug is commercialized as a yellow powder than can be used in aqueous solution up to 10-15 days when stored at room temperature without losing its activity (Fairclough, 1962). DA is recommended as a therapeutic drug at the dosage of 3.5mg/kg body weight (b.w) by intramuscular (IM) route to clear *T. congolense* and *T. vivax* infections in cattle. Standard therapeutic doses of DA (3.5mg/kg b.w) rarely results in signs of toxicity in domestic animals, since the compound is rapidly excreted via urine and feces ($t_{1/2}$ < 1h) (Delespaux and de Koning, 2007; Mamman et al., 1993). However, the product is relatively toxic in dogs and a single dose of 7mg/kg b.w can be highly toxic in camels. Currently, DA is marketed under the trade names of Berenil®, Veriben®, Trypazène®, Trypan®, Ganaseg®, etc. and is widely available and commonly used by farmers in West Africa. Additionally to its availability and low cost, DA is also used to treat animals affected by various tick-borne diseases, including babesiosis (Bruning, 1996). These clear advantages have made of DA a first-line treatment for sick animals.

1.9.3.2. Isometamidium chloride

ISM is a phenanthridinium salt (Browning et al., 1938). Chemically, ISM is known as 8-[(m-amidinophenyl-azo) amino]-3-amino-5-ethyl-6-phenylphenanthridinium chloride hypochloride ($C_{28}H_{25}ClN_7HCl$; MW: 531.5) and differs from homidium (see later) by an additional moiety of m-amidinophenyl-azo-amine (Wragg et al., 1958) (Figure 1.7). In other words, ISM is synthesized by coupling homidium with a part of the diminazene molecule (Delespaux and de Koning, 2007).

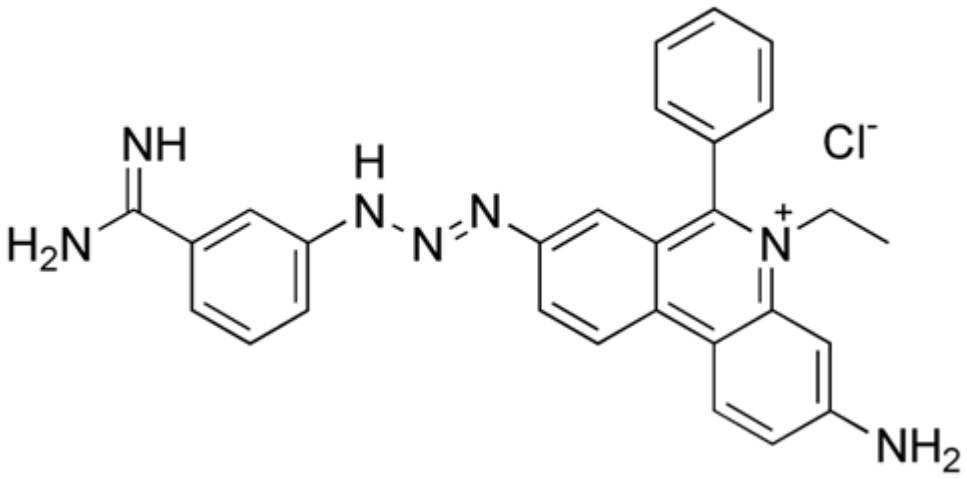

Figure 1.7: Molecular structure of ISM (Delespaux et al., 2010)

ISM is an amphiphilic cationic drug, which is commercialized as a dark reddish-brown powder. ISM is less soluble in pure organic solvents and labile under low and high pH conditions and at a high temperature. Its solubility in water is about 6% (w/v) at 20°C (Kinabo and Bogan, 1988). As marketed (Trypamidium®, Samorin®), ISM product contains 70% of ISM and 30% of a mixture of its two isomers and a small proportion of a bis-compound (bis designates the number of each type of ligand in the complex ion) and homidium (Novidium® and Ethidium®). ISM is used in aqueous solution (1 or 2%) mainly by deep intra muscular route at doses between 0.25 and 1 mg/kg b.w., depending on the risk of TDR. To clear infections with *T. vivax* and *T. congolense* in bovines and small ruminants, the drug is recommended at doses between 0.25 and 0.5 mg/kg b.w.. Moreover, it protects animals that received doses of 0.5 to 1 mg/kg b.w. for a period between 2 to 4 months (Chartier et al., 2000).

1.9.3.3. Homidium salts

Chemically, homidium is a 3,8-diamino-5-ethyl-6-phenylphenanthridinium. Homidium is better known by its chloride salt or Novidium® ($C_{21}H_{20}ClN_3$; MW: 349.86) and its bromide salt or ethidium bromide (Ethidium®; $C_{21}H_{20}BrN_3$; MW: 394.31) (Figure 1.8).

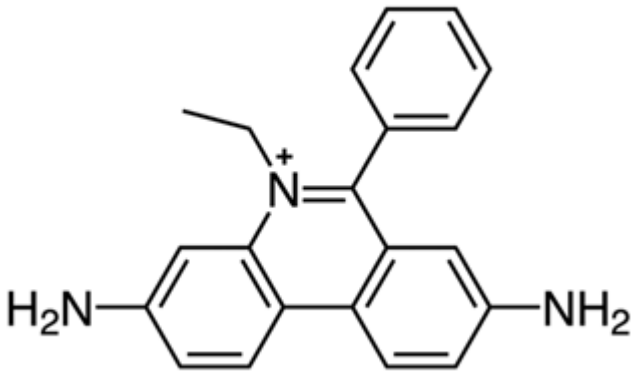

Figure 1.8: Molecular structure of homidium (Delespaux et al., 2010)

Used at the dose of 1 mg/kg b.w. (IM) homidium is active against *T. congolense* and *T. vivax* infections in cattle. The compound is essentially used as a curative drug in the field, even if some studies reported a prophylactic effect varying from 2 to 19 weeks (Dolan et al., 1990; Stevenson et al., 1995). Homidium was widely used during the 1960s but due to the spread of resistance and its mutagenic activity, its use has greatly decreased (Geerts et al., 2010; Kinabo, 1993). The guideline is actually to forbid it for treating animals. This is highly understandable when considering the precautions taken by laboratory technicians when using this compound for DNA staining (Ethidium bromide).

Phenanthridinium drugs exhibits their antitrypanosomal activity through both the blockade of nucleic acid synthesis via DNA intercalation, inhibition of RNA and DNA polymerase and the incorporation of nucleic acid precursors into DNA and RNA (Kinabo, 1993). However, there are others biochemical mechanisms involved in the trypanocidal effect of these drugs, including the modulation of glycoprotein biosynthesis lipid metabolism, membrane transport and selective cleavage of kDNA minicircles (Shapiro and Englund, 1990).

These trypanocidal drugs were used for more than 50 years and have been manufactured for decades by European drug companies. However, recently, cheaper generic forms of these compounds with different levels of quality have flooded all over the African market (Geerts et al., 2010). Unfortunately, due to the limited number of field veterinarians, most livestock keepers treat their sick animals themselves or via

more experienced farmers. They often purchase the trypanocidal drugs from the informal sector (Affognon et al., 2009). Several recent studies have shown that these farmers, besides not having enough knowledge on trypanocidal drugs usage (Delespaux et al., 2002), usually underestimate the body weight of the treated animals and consequently under-dose their animals (Machila et al., 2008). These bad practices in the use of ISM and DA have contributed to the development of TDR in about 17 sub-Saharan African countries (Delespaux et al., 2008a).

1.10. Trypanocidal drug resistance

1.10.1. Definition and etiology

As previously stated, in the context of this thesis, TDR is defined as the decreased or absence of sensitivity of trypanosome strains to standard quality trypanocidal drugs at the dose recommended by the manufacturer and administered according to the good veterinary practice. It is still unclear if TDR is spreading from resistant genotypes existing in wild trypanosome populations that are selected by drug pressure or if it is the drug pressure that is inducing mutations having resistance as a consequence. The fact that it is possible to induce TDR *in vitro* by gradual exposure to the drug (Peregrine et al., 1991) tends to indicate that acquisition of TDR is a reality. However, the isolation of drug resistant trypanosomes from wildlife that were never in contact with the drug (Chitanga et al., 2011) suggests that TDR is existing without any drug pressure and was existing before the discovery of the drugs. A combination of both processes could be considered when we observe the gradation of TDR varying from a slight decrease to a complete loss of sensitivity to the drug (i.e. that the host is killed by the drug before the parasite). This gradation could be explained by the co-existence of different mechanisms of TDR, which are adding their effects to achieve a certain level of resistance.

When the trypanosome is resistant to more than one drug, it is considered as multidrug resistant. In this case, different resistance mechanisms are acquired / selected independently through exposure to different drugs (Black et al., 2001). This is the case for DA and ISM. Cross resistance is a resistance to a particular drug that often results in resistance to another drug, usually from a similar chemical class, to which the trypanosome may not have been exposed. Here, a single mechanism is responsible for resistance to more than one drug. This is the case for quinapyramine that causes

resistance to DA and ISM (Chartier et al., 2000; Uilenberg, 1998). For this reason it has been removed from the market in the seventies. Unfortunately, this drug is currently again available in Africa originating from Asian markets where the drug is allowed. Quinapyramine should be strictly restricted to the treatment of horses and camels infected with *T. evansi*.

Many factors contribute to the development of TDR in West Africa. Indeed, since the 90s and the privatization of the veterinary services, the policy of veterinary medicine restricted the administration of trypanocides to veterinarians and their distribution by registered private veterinarians (Sidibé, 2003). In practice, factors like the few number of private field veterinarians agents associated with the porosity of African borders and the high cost of the formal sector have led to the proliferation of an informal sector with often poor drug quality (Coulibaly, 2004). There is little control on drug sale and an evident lack of information on the correct condition of trypanocides storage and use. All of these factors, associated to the change of farming systems (extensive to more intensive farming in peri-urban zone with increased treatments) and the absence of new products favor the development of TDR (Figure 1.9).

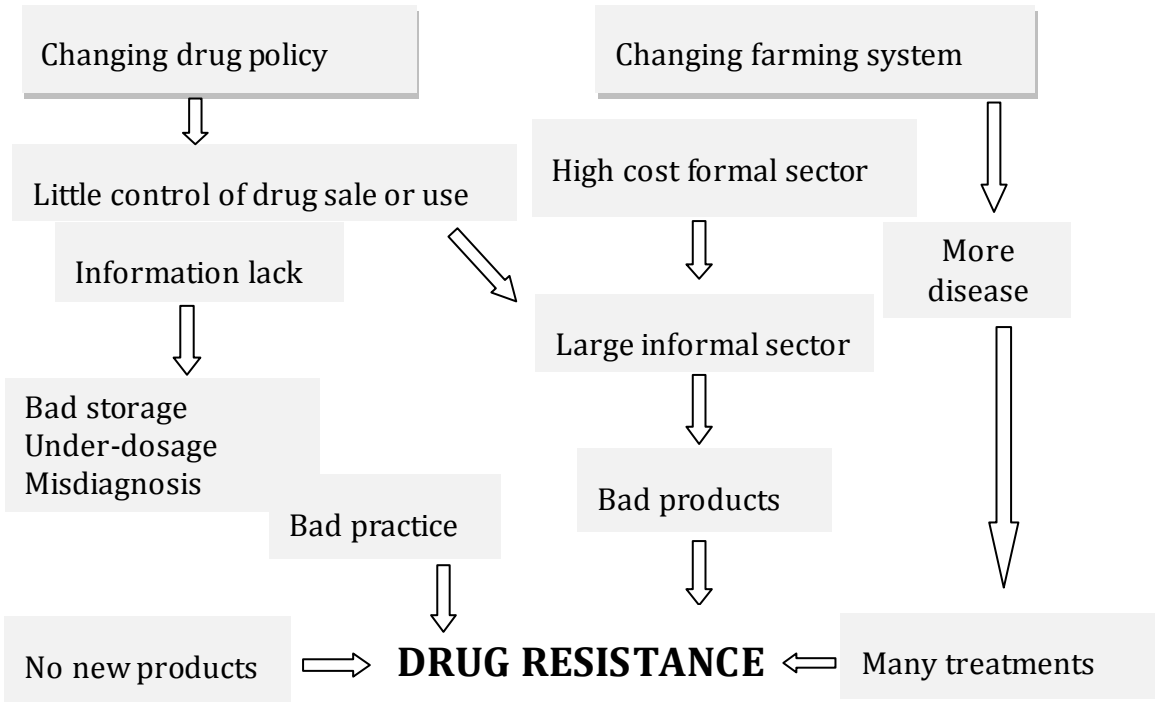

Figure 1.9: Factors fostering TDR in West Africa (Anonymous, 2004)

1.10.2. Distribution

In West Africa, TDR was first identified in 1984 and concerned *T. congolense* resistance against ISM (Authie, 1984). A few years later, multiple resistance was confirmed in the pastoral area of Samorogouan in Burkina Faso (Clausen et al., 1992). More recently, the West Africa's cotton belt was found affected by drug resistance concerning the two main molecules used in this zone i.e. DA and ISM (McDermott et al., 2003; Diall, 2005; Grace, 2005; Talaki et al., 2006; Affognon et al., 2009; Clausen et al., 2010). While, in West Africa, reports of the occurrence of TDR are increasing, it is not really clear whether this is due to a real increase of the TDR problem or just an increased interest by scientists. Nevertheless, a report on a five-fold increase in the prevalence of DA resistance over a seven year period in the Eastern Province of Zambia, suggests that there might be a worsening of the problem (Delespaux et al., 2008b). Currently, TDR has been officially reported in 17 African countries (Figure 1.10) (Delespaux et al., 2008a).

Figure 1.10: Countries for which reports on TDR in animal trypanosomes are available. Adapted from Delespaux et al. (2008a).

However, there are probably more countries affected by TDR. This lack of information is related to the laborious and costly nature of traditional TDR detection tests. Hence, an epidemiological surveillance network for trypanosomosis and TDR (RESCAO) was created in 2009 in West Africa with the goal to monitor the evolution of TDR, especially in zones with suspected multiple drug resistance.

1.10.3. Epidemiological monitoring network of chemoresistance to trypanocidal and acaricides drugs in West Africa (RESCAO)

1.10.3.1. Objectives

Created in April 2009 by the "Centre International de Recherche-Développement sur l'Elevage en zone Subhumide" (CIRDES) of Bobo-Dioulasso and the Institute of Tropical Medicine of Antwerp, the RESCAO is an epidemiological surveillance network with regional ambitions, of which the main objective is to contribute to the improvement of the livestock health and agriculture productivity in West Africa, through effective strategic control of trypanosomosis and tick-borne diseases by a rational use of the available therapeutic arsenal. More specifically, it aims at: (i) a better understanding of the epidemiology of TDR and acaricides in West Africa, (ii) proposing control strategies adapted to the local conditions and (iii) implementing the CIRDES as a West Africa's Regional Center for the molecular diagnosis of both AAT and TDR. This network was funded by the Belgian Development Cooperation (Vitouley et al., 2013).

1.10.3.2. Institutional and structural organization

The membership in RESCAO is institutional and application can be done by sending a letter of commitment signed by the highest authority of the country's center, which in turn will appoint a focal point (Table 1.3) that will remain in permanent contact (quarterly reports activities carried out, annual meeting, etc.) with RESCAO coordination based in the CIRDES. Members are governmental research institutes in animal health and production, universities / training schools in animal health and production, international organizations, etc… To date, the RESCAO includes 8 West African countries (Benin, Burkina Faso, Côte d'Ivoire, Ghana, Mali, Niger, Nigeria and Togo) (Figure 1.11), the interstate school of veterinary sciences and medicine of Dakar (EISMV) via its laboratory of control of the quality of veterinary drugs (LACOMEV), ITM and CIRDES.

Table 1.3: Focal points of RESCAO

Identities	Email addresses	Home institution	Country
POMALEGNI Charles	cpomalegni@yahoo.fr	Institut National de Recherche Agronomiques du Bénin (INRAB)	Bénin
OUATTARA Lassina (DGSV)	sielouattara@hotmail.com	Direction Générale des Services Vétérinaires	Burkina Faso
Mme KOMOIN Clarisse	cl.komoin@gmail.com	Laboratoire National d'Appui au Développement Agricole (LANADA)	Côte d'Ivoire
Alledje-Cudjoe Emmanuel	emmallec@yahoo.com	Central Veterinary Laboratory/Pong-Tamalé	Ghana
DIARRA Boucader	diarrab@gmail.com	PATTEC	Mali
GAMATIE Djibo	gamatieal@hotmail.com	Laboratoire Central de l'Elevage (LABOCEL)	Niger
Mamman Muhammad	mammanm@hotmail.com	Nigerian Institute for Trypanosomiasis Research (NITR)	Nigeria
DAO Balabadi	balabadidao@gmail.com	Institut Togolais de Recherches Agronomiques (ITRA)	Togo

Figure 1.11: West Africa's map
Adapted from http://www.afdb.org/fr/countries/west-africa/
The yellow circle shows the 8 RESCAO's member countries.

Furthermore, as all epidemiological surveillance networks, the modus operandi of the RESCAO can be summarized in four main steps (Dufour and Hendrikx, 2009): (i) data collection, (ii) data transmission, (iii) data management and processing, and (iv) dissemination of the results. Thus the network is structured as a pyramid and is headed by a regional steering committee followed by the regional technical committee, the regional animation cell and national field units.

Based in the CIRDES, the regional steering committee is constituted by the ITM, the CIRDES and the focal points within the countries, universities and others member institutions. This committee decides on the major policies and sets objectives, validates results, assesses progress and settles corrective actions when necessary. The regional technical committee is composed of an epidemiologist in charge of the dynamism of the network and scientists known for their work in the RESCAO's targeted research area. This committee contributes to the design of the protocols and organizes the analysis of collected data. Hosted at the CIRDES, the regional animal cell includes a facilitator and technical assistants specialized in data processing and in the dissemination of the results. The national animation cell is constituted by the focal point assisted by resources persons (veterinarians, veterinary assistants, laboratory technicians, etc.) from the same institution. Its role is to implement procedures and actions defined by the regional steering committee. Finally, field units, decentralized structures of the national cells, are constituted by veterinary stations, research stations, health and animal production schools, veterinary clinics, farmers NGOs, etc (Figure 1.12).

Therefore, to resume Dufour and Hendrikx (2009) criteria's, the RESCAO could be characterized as follow: supranational network, targeted surveillance of drug resistance, active data collection and autonomous from others usual activities.

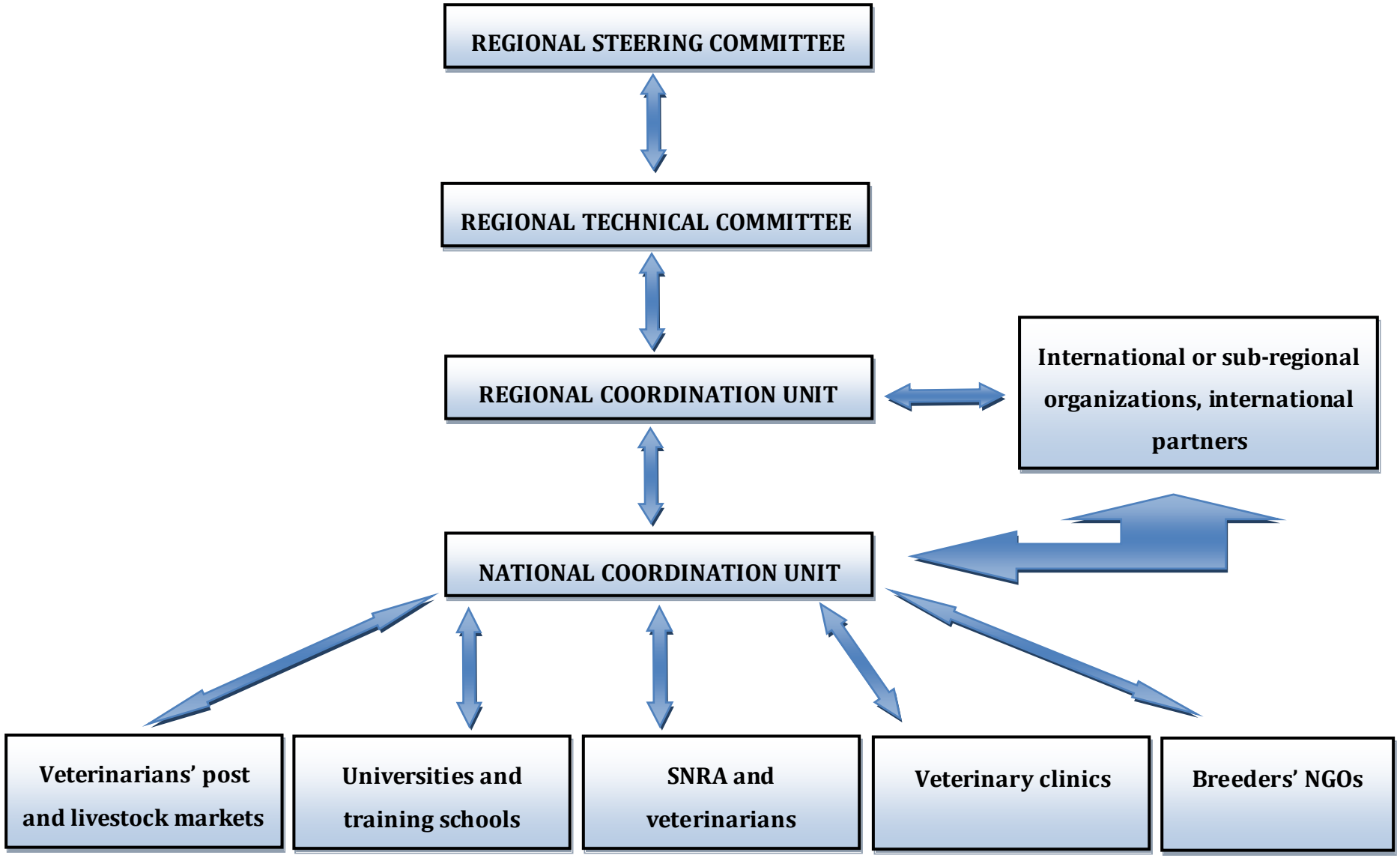

Figure 1.12: The flowchart of the RESCAO

1.10.4. Mechanisms of TDR

1.10.4.1. Isometamidium chloride

ISM is firstly seen in the cytoplasmic compartment of the trypanosome from where the drug is driven to its primary site of accumulation i.e. the kinetoplastic compartment (Wilkes et al., 1995). From the outside environment, ISM is driven down the concentration gradient and enters the cell via a facilitated diffusion, which therefore does not require an expenditure of metabolic energy. Then, ISM is actively transported into the kinetoplastic compartment probably due to the mitochondrial electrical potential (Wilkes et al., 1997) or through an as yet unidentified energy-consuming transmembrane transporter (Delespaux et al., 2005). When placed in an ISM free medium, the diffusion of ISM out of the cell does not vary between sensitive and resistant strains. However, under the same conditions, a large proportion of the drug sequestered within the mitochondrion of the sensitive strains is retained (Wilkes et al., 1997). Therefore, ISM-resistance might probably be caused by the synergistic combination of reduced uptake and increased efflux of the drug at the level of the mitochondrion (Figure 1.13) i.e. (i) a decrease in transport through the mitochondrial membrane (lowered mitochondrial electrical potential), (ii) the modification of a possible transporter located in the inner mitochondrion membrane, (iii) an increased efflux of the drug from the cytoplasmic compartment via a – yet to be identified - transporter or (iv) a combination of these processes (Delespaux et al., 2008a; Sutherland and Holmes, 1993).

Other authors mentioned also the role of a mutation in an ATP-binding-like-transporter as shown by the presence of a conserved *GAA* codon insertion in a gene coding for a putative ABC (ATP Binding Cassette) transporter in the resistant clone (Delespaux et al., 2005). ABC transporters are ubiquitous membrane proteins that use directly the energy from ATP to transport substrate across biomembranes, irrespective of the concentration gradient (Borst and Elferink, 2002). However, more than one resistance mechanism could be involved since some strains characterized as resistant in the mouse test (Eisler et al., 2001) did not show the *GAA* insertion codon (Delespaux et al., 2005).

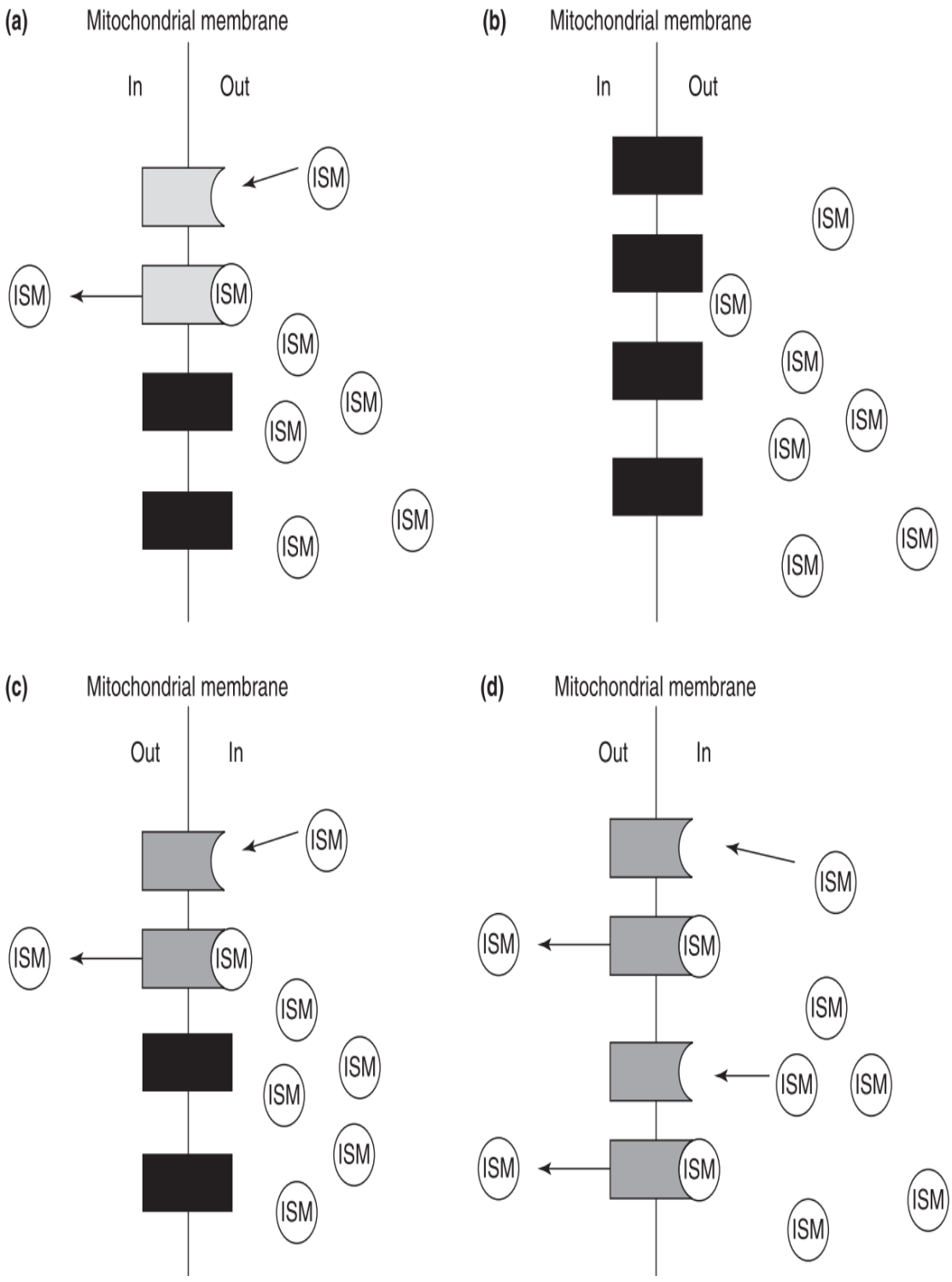

Figure 1.13: Model of the uptake of ISM by *T. congolense* mitochondria.

(a, b) Drug importer models: (a) Heterozygous wild-type (light grey) and mutated (black) importers with decreased activity. (b) Homozygous mutated importers with decreased affinity. Only the homozygous mutated importers will be resistant to ISM.
(c, d) Drug exporter models: (c) Heterozygous wild-type (black) and mutated exporters with increased affinity (dark grey). (d) Homozygous mutated exporters leading to a resistant phenotype. Heterozygous and homozygous mutated exporters would both be resistant to ISM to different degrees (Delespaux et al., 2008a).

ISM resistance mechanism could also include the alteration or modification of the targeted site of the drug since it has been suggested that the main mode of action of ISM was the cleavage of kDNA-topoisomerase complexes (Shapiro and Englund, 1990). The silencing of the mitochondrial topoisomerase gene by RNA interference or by the use of specific topoisomerase II inhibitors induces the progressive shrinking and disappearance of the kinetoplast DNA network (Cavalcanti et al., 2003; Wang and Englund, 2001). This possible mechanism of resistance to ISM (i.e. modification of the topoisomerase II gene) was explored in *T. congolense* (Delespaux et al., 2007). Topoisomerases are a class of enzymes that alter the supercoiling of double-stranded DNA and then serve to maintain both the transcription and replication of DNA. Topoisomerase II cuts both strands of the DNA to relax coil and extend the DNA molecule. The two mitochondrial topoisomerase genes of 10 ISM-sensitive and 14 ISM-resistant strains of *T. congolense* were screened using the single-strand conformation polymorphism (SSCP) technique, sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). This study revealed that both genes were highly conserved and that no polymorphism related to ISM-resistance could be observed (Delespaux et al., 2007).

1.10.4.2. Homidium salts

Contrary to ISM, homidium is spread much more diffuse throughout the trypanosome (Boibessot et al., 2002). Despite their mutagenic activity, Homidium salts (Ethidium® and Novidium®) are still found on the African trypanocidal drug market, less in West than in East Africa (Geerts et al., 2010; Macgregor and Johnson, 1977). The resistance mechanism to this drug is still unknown even if some studies suggested that it is similar to ISM (Peregrine et al., 1997).

1.10.4.3. Diminazene aceturate

Given the highly charged nature of diamidine compounds, specific carriers are required to translocate the drugs across biomembranes. Conversely, the absence or loss of these transport activities would render cells insensitive to this class of drugs, and may explain both their selective toxicity and the probable resistance mechanism. Mediated uptake of DA by *T. brucei* was first demonstrated *in vitro* by Girgis-Takla and James (1974) and found to be lifted after depletion of glucose, indicating then an active uptake. However, it

was after Carter and Fairlamb's (1993) experiment showing the uptake of melarsoprol by the P2 aminopurine transporter, that it emerged that the same transporter might mediate transport of diamidines as well. Indeed, trypanosomes import purines from their hosts through a variety of transporters among which, the adenosine transporters P1 and P2. Whereas P1 was shown to be a broad-specificity purine transporter, P2 is a high affinity transporter for aminopurine, adenine and adenosine (Carter and Fairlamb, 1993; Delespaux et al., 2006). Barret et al. (1995) found that induction of DA resistance in *T. equiperdum* coincided with the loss of P2 activity. Similarly, alterations of the P2-aminopurine transporter encoded by the *TbAT1* gene resulted in DA resistance in *T. b. brucei* (Delespaux and de Koning, 2007). Alteration of P2-transporter activity may not be the only mechanism contributing to DA resistance. Indeed, a novel gene *TeDR40*, the encoded protein appeared to have a ubiquitous cellular localization, is shown to contribute to a high DA resistance in *T. evansi* (Witola et al., 2005). It is probable that such a high level of DA resistance is the result of the cumulative effect of two distinct resistance mechanisms (*TeDR40 and* P2-type purine transporter). Concerning *T. congolense*, a putative P2-type purine transporter *TcoAT1* was identified by blasting the *TbAT1* gene of *T. b. brucei.* A conserved *Val306* to *Ile306* permutation in this gene was observed and showed to be correlated with the DA resistance phenotype (Delespaux et al., 2006). Very recently it has been demonstrated that the *TcoAT1* encoded protein is a P1-type purine transporter that is not involved in DA transport (Munday et al., 2013). Therefore, it was proposed to rename the gene *TcoAT1* into *TcoNT10.* However, a genetic linkage between the observed mutation in the *TcoAT1* gene and DA resistance cannot be excluded as a consistent statistical correlation has been previously demonstrated (Delespaux et al., 2006). A clear trend exists towards homozygosis for this DA-resistance marker in areas of increasing drug usage (Delespaux and de Koning, 2013).

1.10.5. Methods of TDR detection

Several methods have been described to identify drug resistance in trypanosomes. These include field and conventional laboratory tests (*in vivo, in vitro* and ISM-ELISA methods) and more recently molecular tools. With the exception of the recently developed molecular tools, all laboratory tests are time-consuming, laborious and costly because all experimental animals (mice or ruminants) have to be examined twice a week

for the detection of trypanosomes in the peripheral blood. Similarly, in the field test a large number of cattle have to be screened every two weeks over a period of two to three months.

1.10.5.1. Field tests for the detection of TDR

ISM treatment failure can be assessed in the field, under natural *Trypanosoma* challenge, by using the "block treatment" approach (Eisler et al., 2000). It consists of two groups of infected cattle (30 to 80 animals) either treated with 1mg/kg ISM or untreated (control group). The animals are exposed to natural challenge and tested for the presence of trypanosomes in the blood by using the phase contrast buffy coat technique (Murray et al., 1977) every two weeks during two to three months. Drug resistance will be strongly suspected if more than 25% of ISM-treated animals become infected within eight weeks of exposure. A variant of the "block treatment" technique is also used to assess suspected DA and ISM treatments failures by treating both the two groups with DA and ISM respectively at the start of the experiment, and checking for the presence of parasites two weeks after treatment (Mamoudou et al., 2006; McDermott et al., 2003; Rowlands et al., 1993).

1.10.5.2. *In vivo* tests

A standardized protocol has been described to assess the susceptibility and resistance of trypanosomes in mice and ruminants (Eisler et al., 2001). The long duration of these studies (60 days in mice and up to 100 days in ruminants) is the major disadvantage. However, there is a good correlation between the test in mice and the one in ruminants. Nevertheless, the curative dose used in ruminants cannot be extrapolated from the results in mice (Geerts et al., 2000). Finally, the fact that *T. vivax* and some *T. congolense* strains do not grow in mice constitute another limit of these tests.

1.10.5.3. *In vitro* tests

In vitro tests can be used to detect resistance in *T. brucei* and *T. congolense* (Gray et al., 1993; Hirumi et al., 1993). However, the slow adaptation of trypanosomes to the experimental conditions is one of the major constraints of these tests (Clausen et al., 2000). Considering the complexity of maintaining *T. congolense in vitro*, two alternative methods have been developed in which a short *in vitro* incubation in the presence of

various drug concentrations is sufficient. The Drug Incubation Glossina Infectivity Test (DIGIT), which is the first approach, is limited by the availability of tsetse flies (Clausen et al., 1999). Briefly, it consists in feeding flies with blood containing trypanosomes and different concentrations of drugs. The flies are then dissected 20 days later to check their infection status. In the second method, the Drug Incubation Infectivity Test (DIIT), mice are infected by trypanosomes after drug incubation (Knoppe et al., 2006).

1.10.5.4. ISM-ELISA and the mitochondrial electrical potential

For the detection of TDR, several other rarely used tests include the ISM-ELISA technique and a test based on the measurement of the mitochondrial electrical potential (MEP). Wilkes et al. (1997) suggested that the variation of the MEP might be the primary factor determining the rate of ISM accumulation in the trypanosome kinetoplast. Indeed, their initial studies using *T. congolense* populations have shown that an increased or decreased MEP might be a candidate quantitative marker for ISM susceptibility or resistance, respectively. However, the decrease in the MEP could also be related to the interference of a drug extruder generating an important energetic consumption. Actually, the accumulation of ATP in the mitochondrion is causing a high MEP and inversely, a decrease in ATP linked to an important consumption of it will decrease the MEP.

The ISM-ELISA technique can be used in addition to the “block treatment” or individual treatment of ruminants to detect resistant trypanosomes (Eisler et al., 1996). The presence of trypanosomes in animals with an ISM serum concentration > 0.4 ng/ml suggests that parasites are resistant (Eisler et al., 1997).

1.10.5.5. Molecular tools for the detection of TDR

Two molecular tools for the detection of ISM resistance in cattle have been developed. Recent studies using the AFLP technique allowed differentiating two isogenic clones of *T. congolense*, differing in their ISM-sensitivity phenotype. From these results, a PCR-RFLP test using the restriction enzyme *Mbo*II was developed and used to diagnose *T. congolense* resistant to ISM (Delespaux et al., 2005). The test is based on the polymorphism observed in a 381bp fragment (sensitive strains) or 384bp fragment (in resistant strains) of a putative gene presenting some homologies with an ABC

transporter. Indeed, the gene in ISM-resistant strains of *T. congolense* has a conserved triplet insertion (GAA) coding for an extra lysine. The correlation of the *Mbo*II-PCR-RFLP tool with the standard mouse test was 85.7% for *T. congolense* isolates (N=30) collected from different areas of the tsetse fly belt (Delespaux et al., 2005). This number decreased to 60% (Delespaux et al., 2008a) and 75% (Dayo, 2005) when the same test was used on 20 *T. congolense* strains originating from Ethiopia and Burkina Faso and 9 isolates from Zambia, respectively. In a recent study in Cameroon, the *Mbo*II-PCR-RFLP only identified 4 strains as resistant among 12 isolates confirmed to be resistant in the *in vivo* mouse test (Mamoudou et al., 2008). These results suggest the existence of an alternative mechanism to ISM resistance.

Another test, *Sfa*NI-PCR-RFLP, based on the polymorphism of a 677bp fragment of the *Tb*AT1 gene allowed the distinction between ISM-resistant and ISM-sensitive strains of *T. b. brucei* (Afework et al., 2006). However, the authors did not use strains that were specifically mono-resistant to ISM. Unfortunately, from the available data none of these recently developed molecular tools for the detection of ISM-resistance are fully satisfactory. Further field and laboratory work are required. The reduction in cost of whole genome sequencing allows new research strategies i.e. permits to perform more whole genome analysis. The Veterinary Protozoology Unit of the Institute of Tropical Medicine of Antwerp (ITM) is now running whole genome sequencing on field strains (+/- 30) that were previously characterized for their sensitivity/resistance to ISM in the mouse model (Delespaux, pers. Comm.).

Concerning DA-resistance diagnosis, a PCR-RFLP test using the restriction enzyme *Bcl*I was also developed by the ITM. Studies made on 26 resistant *T. congolense* strains coming from various geographic areas and previously characterized for their resistance/sensitivity to DA into mice, have concluded that the *Bcl*I-PCR-RFLP technique is a powerful tool for diagnosing the presence or absence of *T. congolense* resistance to DA (Delespaux et al., 2006). The test is based on a single nucleotide permutation (G to A) observed in the DA-resistant strains that can be easily detected via *Bcl*I restriction of the amplicon. This single point mutation confers a Val306 to Ile306 permutation in the *TcoNT10* gene. As stated before, there is a statistical correlation between the presence of this mutation and the *in vivo* resistance phenotype but it is not the mutation itself that is affecting the transport of DA within the trypanosome. Concerning *T. brucei,* a conserved

set of six point mutations was described in the *TbAT1* gene of melarsoprol-resistant strains (Mäser et al., 1999). Notwithstanding the crucial role of *TbAT1* gene in high levels of resistance to melarsoprol, others factors such as action of the high-affinity pentamidine transporter (HAPT1), the low-affinity pentamidine transporter (LAPT), aquaporines or ABC transporters could be involved (Bridges et al., 2007; Delespaux and de Koning, 2007; Luscher et al., 2006). Finally, further field validation of the *Bcl*I-PCR-RFLP test and the development of such molecular tools for the diagnosis of *T. vivax* resistant strains will be discussed in subsequent chapters.

1.10.6. Strategies for combating TDR

The treatment of livestock with trypanocidal drugs is the most important control strategy for AAT. The effective use of the existing drugs by smallholder crop-livestock farmers in the cotton zone of West Africa is threatened by the development of widespread resistance (Clausen et al., 2010). Therefore, the guidelines recommended by Geerts and Holmes (1998) for rational drug usage and for delaying the development of TDR are still valid (Table 1.4). These strategies include the use of the sanative pair i.e. a pair of curative drugs which could be alternatively used in the field when resistance to either one of them has developed (Whiteside, 1958). The concept was firstly proposed for the DA / Homidium pair and the DA / ISM pair usually used in the field. However, the effectiveness of this strategy may be questioned by the increased field reports from many parts of West Africa of multiple resistant trypanosomes (Clausen et al., 1992; Talaki, 2008; Clausen et al., 2010; Mungube et al., 2012). In their study in the cotton zone of West Africa, Clausen et al. (2010) have suggested that in such circumstances an integrated approach should be applied to prevent and contain TDR: (i) rational drug use information given to farmers, (ii) training farmers and paravets in integrated trypanosomosis control and (iii) training animal care providers to a more specific diagnosis of trypanosomosis to avoid inappropriate treatment. Notwithstanding all the above strategies, it is crucial to monitor the evolution of TDR, especially in areas with suspected multiple drug resistance. It is then important to use molecular tools.

Table 1.4: Guidelines to delay the development of TDR in livestock (Geerts and Holmes, 1998)

1. Avoid sub-therapeutic drug concentrations
2. Use of the sanative pairs (alternation of ISM and DA)
3. Reduce the treatment frequency
    - *Avoid exclusive reliance on trypanocidal drugs*
    - *Integrated drug usage with other control measures*
        - *Trypanotolerant animals*
        - *Tsetse control*
        - *Stimulate host immunity (good nutrition, avoid stress, etc)*
4. Avoid mass treatments
    - *Limit treatment to animal which needs it (PCV<20)*
5. Ban on use of quinapyramine in cattle

1.11. Reference List

Afework, Y., Mäser, P., Etschmann, B., Samson-Himmelstjerna, G., Zessin, K.H., Clausen, P.H., 2006. Rapid identification of isometamidium-resistant stocks of *Trypanosoma b. brucei* by PCR-RFLP. Parasitol. Res. 99, 253-261.

Affognon, H., Coulibaly, M., Diall, O., Grace, D., Randolph, T., Waibel, H., 2009. Gestion de la chimiorésistance dans le cadre de la lutte intégrée contre la Trypanosomose dans la zone cotonnière de l'Afrique de l'Ouest. In. ILRI Research Report, N°17, Nairobi, Kenya. 58p.

Akol, G.W., Murray, M., 1986. Parasite kinetics and immune responses in efferent prefemoral lymph draining skin reactions induced by tsetse-transmitted *Trypanosoma congolense*. Vet. Parasitol. 19, 281-293.

Allsopp, A., Authié, E.M.L., Barrett, M.P., 2004. The Trypanosomiasis. Wallingford, UK: CABI Publishing. 614p.

Anonymous, 2004. Better management of trypanosomosis in the presence of drug resistance. In. ILRI BMZ Project "Improving the management of trypanocide resistance in the cotton zone of West Africa: a coordinated regional study". Workshop Report, Bobo Dioulasso, Kenedougou, Burkina Faso, pp. 1-45.

Authie, E., 1984. Mise en évidence d'une résistance aux trypanocides parmi les souches de *Trypanosoma congolense* récemment isolés au Burkina Faso. Rev. Elev. Méd. Vét. Pays trop. 37, 219-235.

Baral, T.N., 2010. Immunobiology of African Trypanosomes: Need of Alternative Interventions. J. Biomed. Biotechnol. Volume 2010, Article ID 389153, 24 pages. doi:10.1155/2010/389153.

Barrett, M.P., Zhang, Z.Q., Denise, H., Giroud, C., Baltz, T., 1995. A diamidine resistant *Trypanosoma equiperdum* clone contains a P2 purine transporter with reduced substrate affinity. Mol. Biochem. Parasitol. 73, 223-229.

Bengaly, Z., Sidibe, I., Boly, H., Sawadogo, L., Desquesnes, M., 2002a. Comparative pathogenicity of three genetically distinct *Trypanosoma congolense*-types in inbred Balb/c mice. Vet. Parasitol. 105, 111-118.

Bengaly, Z., Sidibe, I., Ganaba, R., Desquesnes, M., Boly, H., Sawadogo, L., 2002b. Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. Vet. Parasitol. 108, 1-19.

Black, S.J., Seed, J.R., Murphy, N.B., 2001. Innate and acquired resistance to African trypanosomiasis. J. Parasitol. 87, 1-9.

Boibessot, I., Turner, C.M., Watson, D.G., Goldie, E., Connel, G., McIntosh, A., Grant, M.H., Skellern, G.G., 2002. Metabolism and distribution of phenanthridine trypanocides in *Trypanosoma brucei*. Acta Trop. 84, 219-228.

Borst, P., Elferink, R.O., 2002. Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. 71, 537-592.

Bourn, D., Reid, R., Rogers, D., Snow, B., Wint, W., 2001. Environmental change and the autonomous control of tsetse and trypanosomosis in Sub-Saharan Africa: case histories from Ethiopia, The Gambia, Kenya, Nigeria and Zimbabwe. Environmental Research Group Oxford Ltd. (ERGO). 248p.

Bouyer, J., Stachurski, F., Gouro, A.S., Lancelot, R., 2009. Control of bovine trypanosomosis by restricted application of insecticides to cattle using footbaths. Vet. Parasitol. 161, 187-193.

Bouyer, J., Stachurski, F., Kabore, I., Bauer, B., Lancelot, R., 2007. Tsetse control in cattle from pyrethroid footbaths. Prev. Vet. Med. 78, 223-238.

Bridges, D.J., Gould, M.K., Nerima, B., Maser, P., Burchmore, R.J.S., de Koning, H.P., 2007. Loss of the high-affinity pentamidine transporter is responsible for high levels of cross-resistance between arsenical and diamidine drugs in African trypanosomes. Mol. Pharmacol. 71, 1098-1108.

Browning, C.H., Morgan, G.T., Robb, J.U.M., Walls, L.P., 1938. The trypanocidal action of certain phenanthridinium compounds. J. Pathol. Bacteriol. 46, 203-204.

Bruning, A., 1996. Equine piroplasmosis, an update on diagnosis, treatment and prevention. Br. Vet. J. 152, 139-151.

Büscher, P., Mumba, N.D., Kabore, J., Lejon, V., Robays, J., Jamonneau, V., Bebronne, N., Van, d., V, Bieler, S., 2009. Improved Models of Mini Anion Exchange Centrifugation Technique (mAECT) and Modified Single Centrifugation (MSC) for sleeping sickness diagnosis and staging. PLoS Negl. Trop Dis. 3, e471.

Buxton, P.A., 1955. The natural history of tsetse flies. An account of the biology of the genus *Glossina* (Diptera). Lewis H.K. & Co Ltd, London. 816p.

Carter, N.S., Fairlamb, A.H., 1993. Arsenical resistant trypanosomes lack an unusual adenosine transporter. Nature 361, 173-176.

Cavalcanti, D., Guimarães, P.L., Fragoso, S.P., De Souza, W., Goldenberg, S., Motta, M.C.M., 2003. The effect of toposiomerase II inhibitors on the kinetoplast ultrastructure of some trypanosomatids. Microsc. Acta 12, 217-218.

Chartier, C., Itard, J., Morel, P.-C., Troncy, P.-M., 2000. Précis de parasitologie vétérinaire tropicale. TEC & DOC, Editions Médicales Internationales. 769p.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J., Delespaux, V., 2011. High Prevalence of Drug Resistance in Animal Trypanosomes without a History of Drug Exposure. PLoS Negl. Trop. Dis. 5 (12): e1454. doi:10.1371/journal.pntd.0001454.

Clausen, P.H., Bauer, B., Zessin, K.H., Diall, O., Bocoum, Z., Sidibe, I., Affognon, H., Waibel, H., Grace, D., Randolph, T., 2010. Preventing and containing trypanocide resistance in the cotton zone of West Africa. Transbound. Emerg. Dis. 57, 28-32.

Clausen, P.H., Leendertz, F.H., Blankenburg, A., Tietjen, U., Mehlitz, D., Sidibe, I., Bauer, B., 1999. A drug incubation *glossina* infectivity test (DIGIT) to assess the susceptibility of *Trypanosoma congolense* bloodstream forms to trypanocidal drugs (Xenodiagnosis). Acta Trop. 72, 111-117.

Clausen, P.H., Pellmann, C., Scheer, A., Tietjen, U., Schares, G., Bauer, B., Peregrine, A.S., Mehlitz, D., 2000. Application of in vitro methods for the detection of drug resistance in trypanosome field isolates. ICPTV Newsletter 2, 9-12.

Clausen, P.H., Sidibe, I., Bassinga, A., Richard, X., Bauer, B., Pohlit, H., 1993. Pathogenesis and pathology of African trypanosomosis in Baoule, N'Dama/Baoule cross bred and Zebu cattle in Burkina Faso. 1. Clinical performance under high natural tsetse challenge. Trop Med. Parasitol. 44, 99-107.

Clausen, P.H., Sidibe, I., Kabore, I., Baeur, B., 1992. Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. Acta Trop. 51 , 229-236.

Connor, R.J., Van den Bossche, P., 2004. African Animal Trypanosomoses. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press Southern Africa, Cape Town, pp. 251-296.

Coulibaly, H., 2004. Organisation des services vétérinaires dans les pays en développement d'Afrique de l'Ouest. Rev. sci. tech. Off. int. Epiz. 23, 361-373.

Courtin, F., Rayaisse, J.B., Tamboura, I., Serdebeogo, O., Koudougou, Z., Solano, P., Sidibe, I., 2010. Updating the northern tsetse limit in Burkina Faso (1949-2009): impact of global change. Int J Environ. Res. Public Health 7, 1708-1719.

Cuisance, D., Itard, J., Desquesnes, M., Frézil, J.-L., 2003. Trypanosomoses: Epidémiologie. In: Principales maladies infectieuses et parasitaires du bétail, Europe et régions chaudes, pp. 1627-1650.

Davison, H.C., Thrusfield, M.V., Husein, A., Muharsini, S., Partoutomo, S., Rae, P., Luckins, A.G., 2000. The occurrence of *Trypanosoma evansi* in buffaloes in Indonesia, estimated using various diagnostic tests. Epidemiol. Infect. 124, 163-172.

Dayo, G.K., 2005. Corrélation entre le test sur souris et la PCR-RFLP pour la détection de la résistance au chlorure d'isometamidium de souches de *Trypanosoma congolense* de différentes pathogénicités. Master thesis. In. Institut de Médicine Tropicale d'Anvers, pp. 1-37.

de La Rocque, S., Michel, J.F., Cuisance, D., De Wispelaere, G., Augusseau, X., Solano, P., Guillobez, S., Arnaud, M., 2001. Le risque trypanosomien: une approche globale pour une décision locale. La librairie du Cirad TA 283/04, Avenue Agropolis 34398 Montpellier Cedex 5 France. 131p.

Delespaux, V., Chitanga, S., Geysen, D., Goethals, A., Van den Bossche, P., Geerts, S., 2006. SSCP analysis of the P2 purine transporter TcoAT1 gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Trop. 100, 96-102.

Delespaux, V., de Koning, H.P., 2007. Drugs and drug resistance in African trypanosomiasis. Drug Resist. Update 10, 30-50.

Delespaux, V., de Koning, H.P., 2013. Transporters in Anti-Parasitic Drug Development and Resistance. In: Trypanosomatid Diseases: Molecular Routes to Drug Discovery. Published by Wiley-VCH Verlag GmbH & Co. KGaA, pp. 335-351.

Delespaux, V., Dinka, H., Masumu, J., Van den Bossche, P., Geerts, S., 2008b. Five fold increase in the proportion of diminazene aceturate resistant *Trypanosoma congolense* isolates over a seven years period in Eastern Zambia. Drug Resist. Update 11, 205-209.

Delespaux, V., Geerts, S., Brandt, J., Elyn, R., Eisler, M.C., 2002. Monitoring the correct use of isometamidium by farmers and veterinary assistants in Eastern Province of Zambia using the isometamidium-ELISA. Vet. Parasitol. 110, 117-122.

Delespaux, V., Geysen, D., Geerts, S., 2007. Point mutations in mitochondrial topoisomerase enzymes of *Trypanosoma congolense* are not involved in isometamidium resistance. Mol. Biochem. Parasitol. 151, 137-140.

Delespaux, V., Geysen, D., Majiwa, P.A.O., Geerts, S., 2005. Identification of a genetic marker for isometamidium chloride resistance in *Trypanosoma congolense*. Int. J. Parasitol. 35, 235-243.

Delespaux, V., Geysen, D., Van den Bossche, P., Geerts, S., 2008a. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol. 24, 236-242.

Delespaux, V., Vitouley, S.H., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Geerts, S., Van den Bossche, P., 2010. Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracycline and enrofloxacin. PLoS Negl. Trop. Dis. 4, 2-8.

Desquesnes, M., Davila, A.M.R., 2002. Applications of PCR-based tools for detection and identification of animal trypansomes: a review and perspectives. Vet. Parasitol. 109, 213-231.

Desquesnes, M., de La Rocque, S., 1995. Comparaison de la sensibilité du test de de Woo et d'un test de détection des antigènes de *Trypanosoma vivax* chez deux moutons expérimentalement infectés avec une souche guyannaise du parasite. Rev. Elev. Méd. Vét. Pays trop. 48, 247-253.

Desquesnes, M., Dia, M.L., 2003. *Trypanosoma vivax*: mechanical transmission in cattle by one of the most common African tabanids, *Atylotus agrestis*. Exp. Parasitol. 103, 35-43.

Desquesnes, M., Holzmuller, P., Lai, D.H., Dargantes, A., Lun, Z.R., Jittaplapong, S., 2013. *Trypanosoma evansi* and Surra: A Review and Perspectives on Origin, History, Distribution, Taxonomy, Morphology, Hosts, and Pathogenic Effects. Biomed. Res. Int. Volume 2013, Article ID 194176, 22 pages. http://dx.doi.org/10.1155/2013/194176.

Desquesnes, M., McLaughlin, G., Zoungrana, A., Davila, A.M., 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int. J. Parasitol. 31, 610-614.

Diall, O., 2005. Gestion de la chimiorésistance dans le cadre de la lutte contre la trypanosomose dans la zone cotonnière de l'Afrique de l'Ouest. Bulletin 9, 1-7.

Dolan, R.B., Okech, G., Alushula, H., Mutugi, M., Stevenson, P., Sayer, P.D., Njogu, A.R., 1990. Homidium bromide as a chemoprophylactic for cattle trypanosomiasis in Kenya. Acta Trop. 47, 137-144.

Dufour, B., Hendrikx, P., 2009. Epidemiological surveillance in animal health. CIRAD, AEEMA, FAO, OIE, p.386.

Eisler, M.C., Brandt, J., Bauer, B., Clausen, P.H., Delespaux, V., Holmes, P.H., Ilemobade, A., Machila, N., Mbwambo, H., McDermott, J., Mehlitz, D., Murilla, G., Ndung'u, J.M., Peregrine, A.S., Sidibe, I., Sinyangwe, L., Geerts, S., 2001. Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. Vet. Parasitol. 97, 171-182.

Eisler, M.C., Dwinger, R.H., Majiwa, P.A.O., Picozzi, K., 2004. Diagnosis and Epidemiology of African Animal Trypanosomiasis. In: The Trypanosomiasis. CABI Publishing Wallingford, Oxfordshire OX10 8DE, UK, pp. 253-267.

Eisler, M.C., Elliott, C.T., Holmes, P.H., 1996. A simple competitive enzyme immunoassay for the detection of the trypanocidal drug isometamidium. Ther. Drug Monit. 18, 73-79.

Eisler, M.C., Gault, E.A., Moloo, S.K., Holmes, P.H., Peregrine, A.S., 1997. Concentrations of isometamidium in the sera of cattle challenged with drug-resistant *Trypanosoma congolense*. Acta Trop. 63, 89-100.

Eisler, M.C., McDermott, J., Mdachi, R.E., Murilla, G.A., Sinyangwe, L., Mubanga, J., Machila, N., Mbwambo, H., Coleman, P., Clausen, P.H., Bauer, B., Sidibe, I., Geerts, S., Holmes, P.H., Peregrine, A.S., 2000. Rapid method for the assessment of trypanocidal drug resistance in the field. Proceedings of the 9th Symposium of the International Society for veterinary epidemiology and economics, Nairobi, paper 353, 1-3.

El-Sayed, N.M., Hegde, P., Quackenbush, J., Melville, S.E., Donelson, J.E., 2000. The African trypanosome genome. Int. J. Parasitol. 30, 329-345.

Fairclough, R., 1962. A summary of the use of Berenil in Kenya. In. Proceedings, 9th meeting of the International Scientific Council for Trypanosomiasis Research, Conakry, Guinea. *CCTA Publication* 88, pp. 81-86.

Field, M.C., Carrington, M., 2009. The trypanosome flagellar pocket. Nat. Rev. Microbiol. 7, 775-786.

Geerts, S., Delespaux, V., Van den Bossche, P., 2010. Drug Resistance in Trypanosomes of Livestock:A Worrying Issue. Bull. Séanc. Acad. R. Sci. Outre-MerMeded. 55, 177-184.

Geerts, S., Holmes, P.H., 1998. Drug management and parasite resistance in bovine trypanosomiasis in Africa. PAAT Technical Scientific Series 1, 1-38.

Geerts, S., Ndung'u, J.M., Murilla, G., Mbwambo, H., Sinyangwe, L., Machila, N., Delespaux, V., Brandt, J., Peregrine, A.S., McDermott, J.J., Holmes, P.H., Eisler, M.C., 2000. In vivo tests for the detection of resistance to trypanocidal drugs: tests in mice and in ruminants. ICPTV Newsletter 2, 6-7.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171-180.

Girgis-Takla, P., James, D.M., 1974. In vitro uptake of isometamidium and diminazene by *Trypanosoma brucei*. Antimicrob. Agents Chemother. 6, 372-374.

Grace, D., 2005. Epidemiology and control of cattle trypanosomosis in villages under risk of trypanocide resistance in West Africa. PhD thesis. In. Institute for Parasitology and International Animal Health, Free University of Berlin, Germany. 201p.

Gray, M.A., Kimarua, R.W., Peregrine, A.S., Stevenson, P., 1993. Drug sensitivity screening in vitro of populations of *Trypanosoma congolense* originating from cattle and tsetse flies at Nguruman, Kenya. Acta Trop. 55, 1-9.

Hajduk, S., Adler, B., Bertrand, K., Fearon, K., Hager, K., Hancock, K., Harris, M., Le, B.A., Moore, R., Pollard, V., 1992. Molecular biology of African trypanosomes: development of new strategies to combat an old disease. Am. J. Med. Sci. 303, 258-270.

Hide, G., Tait, A., 2004. Genetics and Molecular Epidemiology of Trypanosomes. In: The Trypanosomiases. CABI *Publishing* Wallingford, Oxfordshire OX10 8DE, UK, pp. 77-93.

Hilali, M., Abdel-Gawad, A., Nassar, A., Abdel-Wahab, A., Magnus, E., Buscher, P., 2004. Evaluation of the card agglutination test (CATT/*T. evansi*) for detection of *Trypanosoma evansi* infection in water buffaloes (Bubalus bubalis) in Egypt. Vet. Parasitol. 121, 45-51.

Hirumi, H., Hirumi, K., Peregrine, A.S., 1993. Axenic culture of *Trypanosoma congolense* - Application to the detection of sensitivity levels of blood-stream trypomastigotes to diminazene aceturate, homidium chloride, isometamidium chloride and quinapyramine sulfate. J. Protozool. Res. 3, 52-63.

Hoare, C.A., 1970. The mammalian trypanosomes of Africa. In: The African trypanosomiases. George Allen and Unwin, pp. 3-23.

Hoare, C.A., 1972. The trypanosomes of Mammals. A Zoological Monograph. Blackwell Oxford. 749p.

Jensch, H., 1958. über neue typen von guanylverbindungen. 6,134-169. Med. u. Chem. 6, 134-169.

Kalu, A.U., Edeghere, H.U., Lawani, F.A., 1986. Comparison of diagnostic techniques during subclinical single infections of trypanosomiasis in goats. Vet. Parasitol. 22, 37-47.

Kalu, A.U., Oboegbulem, S.I., Uzoukwu, M., 2001. Trypanosomosis in small ruminants maintained by low riverine tsetse population in central Nigeria. Small Rumin. Res. 40, 109-115.

Kamuanga, M.J.B., Somda, J., Sanon, Y., Kagoné, H., Zoundi, J.S., Hitimana, L., 2008. Elevage et marché régional au Sahel et en Afrique de l'Ouest. *Potentialités et défis.* Paris, Club du Sahel et de l'Afrique de l'Ouest/OCDE, 157p.

Kinabo, L.D., 1993. Pharmacology of existing drugs for animal trypanosomiasis. Acta Trop. 54, 169-183.

Kinabo, L.D., Bogan, J.A., 1988. The pharmacology of isometamidium. J. Vet. Pharmacol. Ther. 11, 233-245.

Knoppe, T.N., Bauer, B., McDermott, J., Peregrine, A.S., Mehlitz, D., Clausen, P.H., 2006. Isometamidium sensitivity of *Trypanosoma congolense* stocks from cattle in West Africa tested in mice and the drug incubation infectivity test. Acta Trop. 97, 108-116.

Krafsur, E.S., 2009. Tsetse flies: Genetics, evolution, and role as vectors. Infect. Genet. Evol. 9, 124-141.

Larsen, T.B., 2008. Forest butterflies in West Africa have resisted extinction...so far (*Lepidoptera*: *Papilionoidea and Hesperioidea*). Biodivers. Conserv. 17, 2833-2847.

Leak, S.G.A., 1999. Tsetse Biology and Ecology. Their role in the epidemiology and control of trypanosomiasis. CABI *Publishing* Wallindford, OX10 8DE, UK. 155p.

Lumsden, W.H., Kimber, C.D., Evans, D.A., Doig, S.J., 1979. *Trypanosoma brucei:* Miniature anion-exchange centrifugation technique for detection of low parasitaemias: Adaptation for field use. Trans. R. Soc. Trop. Med. Hyg. 73, 312-317.

Luscher, A., Nerima, B., Mäser, P., 2006. Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 150, 364-366.

Macgregor, J.T., Johnson, I.J., 1977. Invitro metabolic activation of ethidium bromide and other phenanthridinium compounds - Mutagenic activity in *Salmonella typhimurium*. Mutat. Res. 48, 103-107.

Machila, N., Fevre, E.M., Maudlin, I., Eisler, M.C., 2008. Farmer estimation of live bodyweight of cattle: Implications for veterinary drug dosing in East Africa. Prev. Vet. Med. 87, 394-403.

Magez, S., Caljon, G., Tran, T., Stijlemans, B., Radwanska, M., 2010. Current status of vaccination against African trypanosomiasis. Parasitology 137, 2017-2027.

Magez, S., Radwanska, M., 2009. African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. Future Microbiology 4, 1075-1087.

Mamman, M., Aliu, Y.O., Peregrine, A.S., 1993. Comparative pharmacokinetics of diminazene in noninfected Boran (Bos indicus) cattle and Boran cattle infected with *Trypanosoma congolense*. Antimicrob. Agents Chemother. 37, 1050-1055.

Mamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S., 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. Acta Trop. 106, 115-118.

Mamoudou, A., Zoli, A., Tanenbe, C., Andrikaye, J.P., Bourdanne, A., Delespaux, V., Clausen, P.H., Geerts, S., 2006. Evaluation de la résistance aux produits trypanocides sur le plateau de l'Adamaoua au Cameroun en utilisant un test de terrain et le test standardisé sur souris. Rev. Elev. Méd. Vét. Pays trop. 59, 11-16.

Mäser, P., Sutterlin, C., Kralli, A., Kaminsky, R., 1999. A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. Science 285, 242-244.

Masumu, J., Marcotty, T., Geysen, D., Geerts, S., Vercruysse, J., Dorny, P., Van den Bossche, P., 2006. Comparison of the virulence of *Trypanosoma congolense* strains isolated from cattle in a trypanosomiasis endemic area of eastern Zambia. Int. J. Parasitol. 36, 497-501.

McDermott, J., Woitag, T., Sidibe, I., Bauer, B., Diarra, B., Ouedraogo, D., Kamuanga, M., Peregrine, A.S., Eisler, M.C., Zessin, K.H., Mehlitz, D., Clausen, P.H., 2003. Field studies of drug-resistant cattle trypanosomes in Kenedougou Province, Burkina Faso. Acta Trop. 86, 93-103.

Melville, S.E., Leech, V., Gerrard, C.S., Tait, A., Blackwell, J.M., 1998. The molecular karyotype of the megabase chromosomes of *Trypanosoma brucei* and the assignment of chromosome markers. Mol. Biochem. Parasitol. 94, 155-173.

Melville, S.E., Leech, V., Navarro, M., Cross, G.A., 2000. The molecular karyotype of the megabase chromosomes of *Trypanosoma brucei* stock 427. Mol. Biochem. Parasitol. 111, 261-273.

Melville, S.E., Majiwa, P.A.O., Tait, A., 2004. The African Trypanosome Genome. In: Maudlin, I., Holmes, P.H., Miles, M.A. (Eds.), The Trypanosomiasis. CABI Publishing Wallingford, Oxfordshire OX10 8DE, UK, pp. 39-57.

Munday, J.C., Rojas Lopez, K.E., Eze, A.A., Delespaux, V., Van Den Abbeele, J., Rowan, T., Barrett, M.P., Morrison, L.J., de Koning, H.P., 2013. Functional expression of *TcoAT1* reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake. Int. J. Parasitol.: Drugs and drug resistance 3, 69-76.

Mungube, E.O., Vitouley, H.S., Cudjoe, E.A., Diall, O., Boucoum, Z., Diarra, B., Sanogo, Y., Randolph, T.F., Bauer, B., Zessin, K.H., Clausen, P.H., 2012. Detection of multiple drug-resistant *Trypanosoma congolense* populations in village cattle of south-east Mali. Parasit. Vectors. 5, 155.

Murray, M., Murray, P.K., Mcintyre, W.I.M., 1977. Improved parasitological technique for diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 71, 325-326.

Myler, P.J., 1993. Molecular variation in trypanosomes. Acta Trop. 53, 205-225.

Nakayima, J., Nakao, R., Alhassan, A., Sugimoto, C., 2012. Molecular epidemiological studies on animal trypanosomiases in Ghana. Parasit. Vectors. 5, 217.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R., Saigar, N., Minja, S.H., 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense*, *T. vivax* and *T. brucei*. Parasite Immunol. 9, 421-431.

Ngaira, J.M., Bett, B., Karanja, S.M., Njagi, E.N., 2003. Evaluation of antigen and antibody rapid detection tests for *Trypanosoma evansi* infection in camels in Kenya. Vet. Parasitol. 114, 131-141.

Njiru, Z.K., Constantine, C.C., Ndung'u, J.M., Robertson, I., Okaye, S., Thompson, R.C., Reid, S.A., 2004. Detection of *Trypanosoma evansi* in camels using PCR and CATT/*T. evansi* tests in Kenya. Vet. Parasitol. 124, 187-199.

Nyeko, J.H.P., Olemoiyol, O.K., Majiwa, P.A.O., Otieno, L.H., Ociba, P.M., 1990. Characterization of trypanosome isolates from cattle in Uganda using species-specific DNA probes reveals predominance of mixed infections. Insect Science and Its Application 11, 271-280.

Paris, J., Murray, M., McOdimba, F., 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. Acta Trop. 39, 307-316.

Peacock, L., Cook, S., Ferris, V., Bailey, M., Gibson, W., 2012. The life cycle of *Trypanosoma* (*Nannomonas*) *congolense* in the tsetse fly. Parasit. Vectors. 5, 109.

Peregrine, A.S., Gray, M.A., Moloo, S.K., 1997. Cross-resistance associated with development of resistance to isometamidium in a clone of *Trypanosoma congolense*. Antimicrob. Agents Chemother. 41, 1604-1606.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K., Murphy, N.B., 1991. Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense*. Parasitology 102 Pt 1, 93-100.

Peregrine, A.S., Mamman, M., 1993. Pharmacology of diminazene: a review. Acta Trop. 54, 185-203.

Renard, J.F., Cheikh, L., Knips, V., 2004. L'élevage et l'intégration régionale en Afrique de l'Ouest. Ministère des Affaires Etrangères, FAO-CIRAD, 37p.

Rowlands, G.J., Mulatu, W., Authie, E., Dieteren, G.D.M., Leak, S.G.A., Nagda, S.M., Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe Valley, Southwest Ethiopia . Factors associated with variations in trypanosome prevalence,

incidence of new infections and prevalence of recurrent infections. Acta Trop. 53, 135-150.

Shapiro, T.A., Englund, P.T., 1990. Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. Proc. Natl. Acad. Sci. U. S. A. 87, 950-954.

Shapiro, T.A., Englund, P.T., 1995. The structure and replication of kinetoplast DNA. Annu. Rev. Microbiol. 49, 117-143.

Sidibé, A.S., 2003. Etats des lieux des services vétérinaires d'Afrique. In: Organisation des Services vétérinaires et sécurité sanitaire des aliments. Actes du Séminaire, 27-28 septembre 2002, Tunis (V. Bellemain & E. Mallet, édit.).OIE (Organisation mondiale de la santé animale), Paris, 188 pp.

Smith, J.W., Agyemang, K., Hailu, Z., Jabbar, M., Larbi, A., Osuji, P., 1996. Le développement du secteur laitier en Afrique occidentale: situation actuelle et questions liées au développement ultérieur. In: Politique pour le développement de l'élevage dans les zones humide et subhumide de l'Afrique subsaharienne, Acte du seminaire d'Abidjan, Côte d'Ivoire du 05 au 09 février 1996, Wageningen, CTA.

Soltys, M.A., 1963. Immunity in African trypanosomiasis. Bull. World Health Organ. 28, 753-761.

Stephen, L.E., 1986. Trypanosomiasis: a veterinary perspective. Pergamon Press, UK. (ISBN 0-08-032017-1). 551 p.

Stevens, J.R., Brisse, S., 2004. Systematics of Trypanosomes of Medical and Veterinary Importance. In: The trypanosomiasis. CABI Publishing Wallingford, Oxfordshire OX10 8DE, UK, pp. 1-23.

Stevenson, P., Sones, K.R., Gicheru, M.M., Mwangi, E.K., 1995. Comparison of isometamidium chloride and homidium bromide as prophylactic drugs for trypanosomiasis in cattle at Nguruman, Kenya. Acta Trop. 59, 77-84.

Stijlemans, B., Caljon, G., Natesan, S.K., Saerens, D., Conrath, K., Perez-Morga, D., Skepper, J.N., Nikolaou, A., Brys, L., Pays, E., Magez, S., Field, M.C., De, B.P., Muyldermans, S., 2011. High affinity nanobodies against the *Trypanosoma brucei* VSG are potent trypanolytic

agents that block endocytosis. PLoS Pathog. 7(6): e1002072. doi:10.1371/journal.ppat.1002072.

Sutherland, I.A., Holmes, P.H., 1993. Alterations in drug transport in resistant *Trypanosoma congolense*. Acta Trop. 54, 271-278.

Tabel, H., Wei, G., Shi, M., 2008. T cells and immunopathogenesis of experimental African trypanosomiasis. Immunol. Rev. 225, 128-139.

Talaki, E., 2008. Etude de la résistance des trypanosomes à l'isométamidium et au diminazène dans la zone cotonnière de l'Afrique de l'Ouest (Mali - Guinée - Burkina Faso). In. Université Polytechnique de Bobo-Dioulasso / Institut de Développement Rural / Département d'Elevage, pp. 1-160.

Talaki, E., Sidibe, I., Diall, O., Grace, D., Barry, A.M., Djiteye, A., Bocoum, Z., Clausen, P.H., Randolph, T., Affognon, H., Hendrickx, G., Pangui, L.J., Belem, A.M., 2006. Répartition spatiale des trypanosomoses animales en relation avec la chimiorésistance dans la zone cotonnière de l'Afrique de l'Ouest (Mali et Guinée). Revue Africaine de Santé et de Productions Animales 4, 45-50.

Taylor, K., Authié, E.M.L., 2004. Pathogenesis of Animal Trypanosomiasis. In: The Trypanosomiasis. CABI Publishing Wallingford, Oxfordshire OX10 8DE, UK, pp. 331-353.

Terblanche, J.S., Clusella-Trullas, S., Deere, J.A., Chown, S.L., 2008. Thermal tolerance in a south-east African population of the tsetse fly *Glossina pallidipes* (Diptera, Glossinidae): Implications for forecasting climate change impacts. J. Insect Physiol. 54, 114-127.

Tran, T., Claes, F., Verloo, D., De, G.H., Buscher, P., 2009. Towards a new reference test for surra in camels. Clin. Vaccine Immunol. 16, 999-1002.

Uilenberg, G., 1998. A field guide for the diagnosis, treatment and prevention of African Animal Trypanosomosis. Food and Agriculture Organization of the United Nations, Rome. (ISBN 92-5-104238-1). 157p.

Van Den Abbeele, J., Claes, Y., van Bockstaele, D., Le Ray, D., Coosemans, M., 1999. *Trypanosoma brucei spp*. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118 (Pt 5), 469-478.

Van den Bossche, P., Chigoma, D., Shumba, W., 2000a. The decline of anti-trypanosomal antibody levels in cattle after treatment with trypanocidal drugs and in the absence of tsetse challenge. Acta Trop. 77, 263-270.

Van den Bossche, P., Chitanga, S., Masumu, J., Marcotty, T., Delespaux, V., 2011. Virulence in *Trypanosoma congolense* Savannah subgroup. A comparison between strains and transmission cycles. Parasite Immunol. 33, 456-460.

Van den Bossche, P., de La Rocque, S., Hendrickx, G., Bouyer, J., 2010. A changing environment and the epidemiology of tsetse-transmitted livestock trypanosomiasis. Trends Parasitol. 26, 236-243.

Van den Bossche, P., Delespaux, V., 2011. Options for the control of tsetse-transmitted livestock trypanosomosis. An epidemiological perspective. Vet. Parasitol. 181, 37-42.

Van den Bossche, P., Mudenge, D., Mubanga, J., Norval, A., 1999. The parasitological and serological prevalence of tsetse-transmitted bovine trypanosomosis in the Eastern Caprivi (Caprivi District, Namibia). Onderstepoort J. Vet. Res. 66, 103-110.

Van den Bossche, P., Shumba, W., Makhambera, P., 2000b. The distribution and epidemiology of bovine trypanosomosis in Malawi. Vet. Parasitol. 88, 163-176.

Vitouley, H.S., Bengaly, Z., Adakal, H., Sidibe, I., Van Den Abbeele, J., Delespaux, V., 2013. Réseau d'EpidémioSurveillance de la Chimiorésistance aux trypanocides et aux acaricides en Afrique de l'Ouest (RESCAO). Tropicultura 31, 205-212.

Whiteside, E.F., 1958. The maintenance of cattle in tsetse-infected country. A summary of four years experience in Kenya. In. Proceedings of the Seventh Meeting of the International Scientific Council for Trypanosomiasis Research, CCTA publication No.41, Bruxelles, Belgium, pp. 83-90.

Wilkes, J.M., Mulugeta, W., Wells, C.W., Peregrine, A.S., 1997. Modulation of mitochondrial electrical potential: A candidate mechanism for drug resistance in African trypanosomes. Biochem. J. 326, 755-761.

Wilkes, J.M., Peregrine, A.S., Zilberstein, D., 1995. The accumulation and compartmentalization of isometamidium chloride in *Trypanosoma congolense*, monitored by its intrinsic fluorescence. Biochem. J. 312, 319-327.

Winrock International, 1992. Assessment of animal agriculture in sub-Saharan Africa. Morrilton, Arkansas, USA, Winrock International, 125p.

Witola, W.H., Tsuda, A., Inoue, N., Ohashi, K., Onuma, M., 2005. Acquired resistance to berenil in a cloned isolate of *Trypanosoma evansi* is associated with upregulation of a novel gene, TeDR40. Parasitology 131, 635-646.

Woo, P.T.K., 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. Acta Trop. 27, 384-386.

Wang, Z.F., Englund, P.T., 2001. RNA interference of a trypanosome topoisomerase II causes progressive loss of mitochondrial DNA. EMBO J. 20, 4674-4683.

Wragg, W.R., Washbourn, K., Brown, K.N., Hill, J., 1958. Metamidium: a new trypanocidal drug. Nature 182, 1005-1006.

Objectives of the thesis

This thesis aims at a better insight on the problem of TDR in West Africa by using molecular tools and to propose alternative methods for the management of this phenomenon.

To achieve the above general objective, the study included the following specific objectives:

1. To improve the molecular tools used to diagnose TDR by:

    - Increasing the performance of the existing molecular tool for the detection of DA resistance in *T. congolense* under field conditions using filter papers for sample storage;
    - Prospecting the development of a molecular diagnostic tool for *T. vivax* resistance to DA in the field.

2. To evaluate the impact of TDR on the health status of *T. vivax* infected animals.
3. To explore possibilities to circumvent TDR using alternative treatment i.e. potentiate the efficacy of ISM by combining it with two affordable veterinary antibiotics (Tetracyclines and Enrofloxacin).

Chapter 2

Improved PCR-RFLP for the diagnosis of diminazene aceturate resistance in *Trypanosoma congolense* in the field using filter papers for sample storage.

Adapted from

Vitouley H.S., Mungube E.O., Alledye-Cudjoe E., Diall O., Bocoum Z., Diarra B., Randolph T.F., Baeur B., Clausen P-H., Geysen D., Sidibé I., Bengaly Z., Van den Bossche P. and Delespaux V. (2011). Improved PCRF-RFLP for the Detection of Diminazene Resistance in *Trypanosoma congolense* under Field Conditions Using Filters Papers for Sample Storage. *PLoS Negl Trop Dis* 5(7): e1223. doi:10.1371/journal.pntd.0001223.

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Vitouley, H.S., Bengaly, Z., Adakal, H., Sidibe, I., Van Den Abbeele, J., Delespaux, V., 2013. Réseau d'EpidémioSurveillance de la Chimiorésistance aux trypanocides et aux acaricides en Afrique de l'Ouest (RESCAO). TROPICULTURA 31, 205-212.

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2.1. Introduction

The different methods for the detection of TDR (Eisler et al., 2001; Eisler et al., 2000) and their main inconvenience (labour intensive, time-consuming, costly, etc) were reviewed in the first chapter of this thesis. To address these important drawbacks, the former Department of Animal Health (currently incorporated in the Department of Biomedical Sciences) of the ITM / Antwerp has developed a *Bcl*I-PCR-RFLP test for the molecular diagnosis of DA resistance in *T. congolense* (Delespaux et al., 2006). Although the test performs very well under laboratory conditions (Mamoudou et al., 2008), it required further adaptation and evaluation for use under field conditions with biological material that is not always collected or stored in optimal conditions. More specifically, the test's ability to amplify low concentrations of parasite DNA (as a result of often low parasitaemia in livestock) needs to be enhanced and its specificity improved by preventing incomplete digestion of the amplicons by the *Bcl*I enzyme. This incomplete digestion creates a mixture of undigested and digested amplicons and as such, falsely mixed RFLP profiles (sensitive and resistant). To address those weak points, the following changes were made to the standard protocol: (i) the *Bcl*I restriction enzyme was replaced by a more effective cutter i.e. *Dpn*II restriction enzyme and (ii) a step of whole genome amplification was performed on samples that were found negative in the PCR for species determination.

2.2. Material and methods

2.2.1. Material

449 whole blood spots (40–50 µl) on filter papers were used (Figures 2.1 and 2.2). These were collected from parasitologically positive cattle originating from the cotton zone of South-East Mali where treatments failures to DA and ISM were previously reported (Mungube et al., 2012). The samples were stored sun-protected for 1 year at tropical ambient temperature without adding dehydrated silica crystals in the storage plastic bags (i.e., stored under sub-optimal conditions as it is often the case in rural environments), allowing for potential degradation of the DNA.

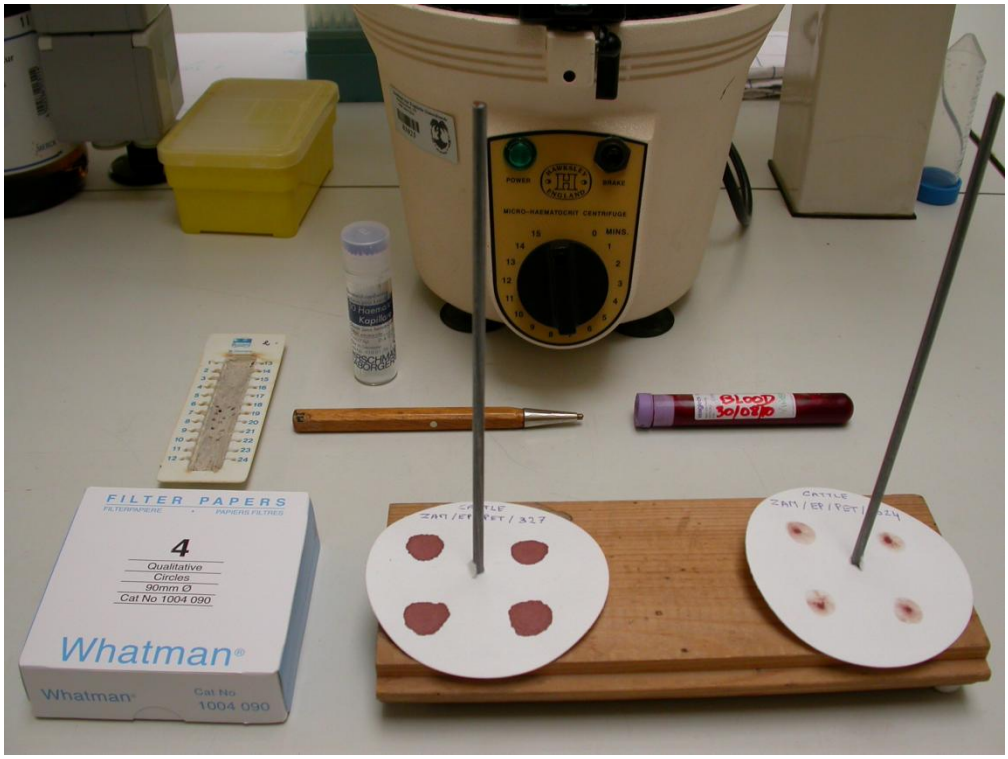


Figure 2.1: Laboratory material used for the processing of the samples.

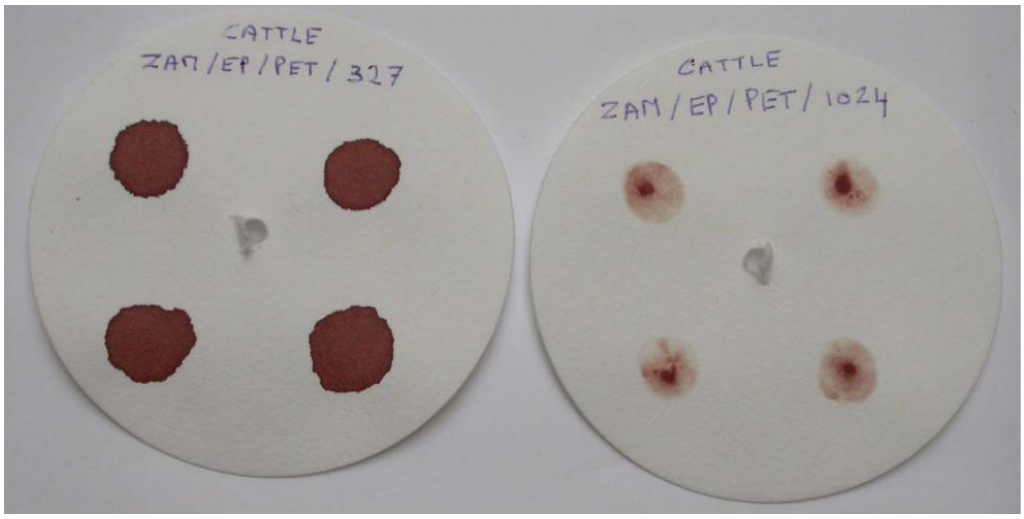


Figure 2.2: Dried blood spots (left) and buffy coats (right) on labelled filter papers.

2.2.2. Methods

2.2.2.1. DNA extraction

DNA extraction was performed following the PBS-Saponin technique (de Almeida et al., 1997). In brief, filter papers discs containing the entire buffy coat or blood spot were cut using a scalpel blade, which was flame-heated between each sample. Each cutting was transferred to a 1.5 ml Eppendorf tube, containing 1ml of PBS-Saponine 0.5%. The tube with the disc was manually mixed and incubated for a minimum of 4h at 4°C or overnight. After a 1 min centrifuging at 15,000 x g, the supernatant was removed and 1 ml of PBS was added to each tube and mixed manually. Followed 1-2 h incubation at 4°C and a novel 1 min centrifugation at 15,000 x g. Afterwards, the maximum of the supernatant were removed without disturbing the pellet. 100 µl of a 10% Chelex-100 resin suspension (Bio-Rad, Herculex, CA) in ultrapure water was added to each tube and incubated for 10 min at 95°C. Thereafter, the samples were centrifuged at 14,000 rpm for 5 min. Finally, the maximum of the supernatant was transferred into a new 1.5 ml Eppendorf tube and stored at -20°C until used.

2.2.2.2. DNA amplification for the detection of trypanosomes

DNA amplification was performed using three primers targeting the 18S small ribosomal subunit gene as described by Geysen et al. (2003) for the diagnosis of trypanosome infections. Thus, standard PCRs were carried out in 25 µl reaction mixtures containing 5 µl DNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µl of each dNTP, 20 pmol of each primer (18STnF2 and 18STnR3) and 0.5 U Taq polymerase enzyme (Goldstar, Eurogentec). The reaction mixture was overlaid by 50 µl fine neutral mineral oil (Sigma) and placed on a heating block of a programmable thermocycler (PTC-100 TM, M.J. Research Inc.; Techgene, Techne Duxford, Cambrigde, UK). After a denaturation step of 4 min at 94°C, each of the 40 cycles consisted of 30s at 92°C, 45s at 58°C and 60s at 72°C. Semi-nested runs was performed adding 0.5 µl of amplification product from the first run to 24.5 µl of PCR mix at 84°C (hot start principle), containing the same ingredients and concentrations except for the primer pair (18ST nF2 and 18ST nR2). The amplification program was identical but only for 25 cycles. A positive and a negative control consisting of adding a reference trypanosome DNA and ultrapure water respectively, were included in each PCR amplification.

In order to observe the presence of DNA fragments within the mixtures, a 5 µl volume of each sample was electrophoresed in a 2% agarose gel for 20 min at 100 V and stained with ethidium bromide for 30 min. A 100bp DNA ladder (MBI Fermentas Lithuania) was included in each gel. Next, the gel was washed under running tap water and photographed under UV illumination.

Sequence of the 5'-3' primers

First round: 18ST nF2 (CAACGATGACACCCATGAATTGGGGA)
18ST nR3 (TGCGCGACCAATAATTGCAATAC)

Semi-nested: 18ST nF2 (CAACGATGACACCCATGAATTGGGGA)
18ST nR2 (GTGTCTTGTTCTCACTGACATTGTAGTG)

After the above detection of *Trypanosoma* spp. infections, RFLP was used for species diagnosis (Geysen et al., 2003).

2.2.2.3. RFLP for species determination

The nested products from the previously described PCR were digested with *Msp*I enzyme in NEBuffer 2 (New England BioLabs) according to the manufacturer's specification (MBI Fermentas, Lithuania) using 10 U/µg DNA (0.6 U/µl PCR product) on 6 µl of amplified DNA in 15 µl total volume. The reaction was left overnight in a water bath at 37°C. A volume of 4 µl of restricted sample was then mixed with 2 µl of loading buffer and transferred onto a 10% polyacrylamide gel together with a 100 bp DNA ladder (MBI Fermentas, Lithuania) for fragment size determination. DNA fragments were thereafter separated by horizontal electrophoresis in 0.5 x TBE buffer at 100V for 2.5 h. The gel was stained using commercial silver staining kit (Silver staining kit DNA plusone, Pharmacia Biotech, Uppsala, Sweden) and mounted for storage.

2.2.2.4. PCR-RFLP for detecting DA resistance

For discriminating the DA sensitive and resistant *T. congolense* genotypes, the following PCR targeting the *T. congolense* putative gene (TcoAT1/TcoNT10) coding for a putative P2-like nucleoside transporter, was used as described by Delespaux et al. (2006). Standard PCR amplifications were carried out as described above except for the couple of primers used i.e. *Ade*2F as forward primer (ATAATCAAAGCTGCCATGGATGAAG) and *Ade*2R (GATGACTAACAATATGCGGGCAAAG) as reverse primer. The PCR products were

then enzymatically restricted as described above, except for the enzyme used. The *Bcl*I restriction enzyme (T^GATCA) was replaced by *Dpn*II (^GATC) to avoid partial digestion of the amplicon. Internal negative and positive controls (sequenced resistant and sensitive strains) were added to ensure the absence of contamination, an effective DNA amplification and the complete digestion of the PCR product. For the visualization of the restriction fragments use was made either of a 4% agarose gel and a staining with ethidium bromide (figure 2.5) or a polyacrylamide gel and a silver staining as described for the species diagnosis (figure 2.6.).

2.2.2.5. Whole genome amplification

Whole genome amplification was performed on the negative samples using the QIAGEN REPLI-g® UltraFast Mini Kit exactly as described by the manufacturer. This kit uses the Multiple Displacement Amplification (MDA) technology that provides high quality and sufficient yield of DNA products for genomic analysis. For economical reasons, the whole genome amplification was not performed on all samples but only on the PCR negatives.

2.3. Results and discussion

2.3.1. Output of the PCR and RFLP for species determination

A total of 68% (304) of all blood spots was found positive for the presence of trypanosomes using the 18S pan-PCR developed by Geysen et al. (2003) (Figure 2.3). Of these, 74% (225) were diagnosed as a single or mixed *T. congolense* savannah (Figure 2.4). The prevalence of the other trypanosome species is not shown since the focus here was the diagnosis of DA-resistance in *T. congolense.* Otherwise, as observed in the figure 2.4, it was impossible to differentiate *T. simiae* species from those of *T. theileri* using *Msp*I as restriction enzyme. However, this has no consequences for epidemiological surveys in cattle since *T. theileri* is not pathogenic for cattle and *T. simiae* does usually not occur in these animals. It would be an important issue when dealing with AAT in small ruminants, which are indeed susceptible for *T. simiae* (Geysen et al., 2003; Stephen, 1986). However if needed, these two species can be differentiated by the *Mbo*II enzyme on the same amplicon (Geysen et al., 2003).

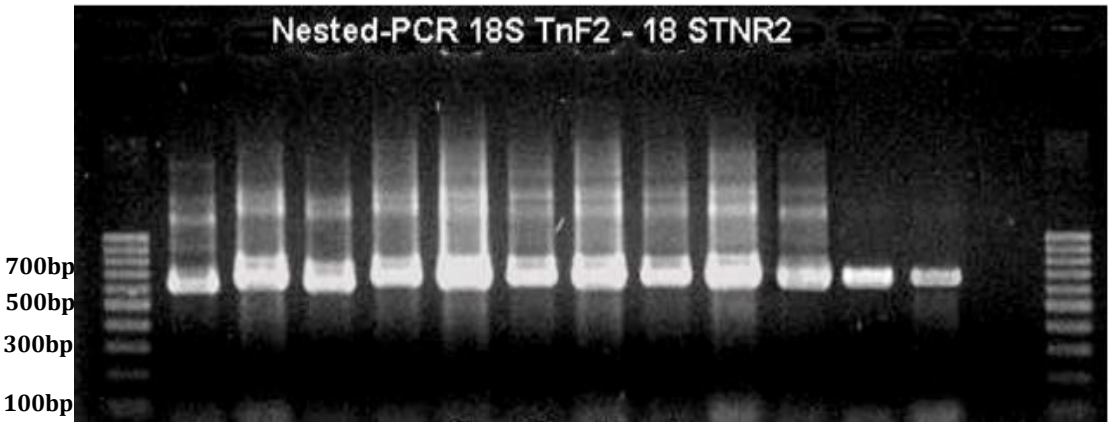


Figure 2.3: Nested PCR 18S profiles of the samples (*Trypanosoma* spp. infections)

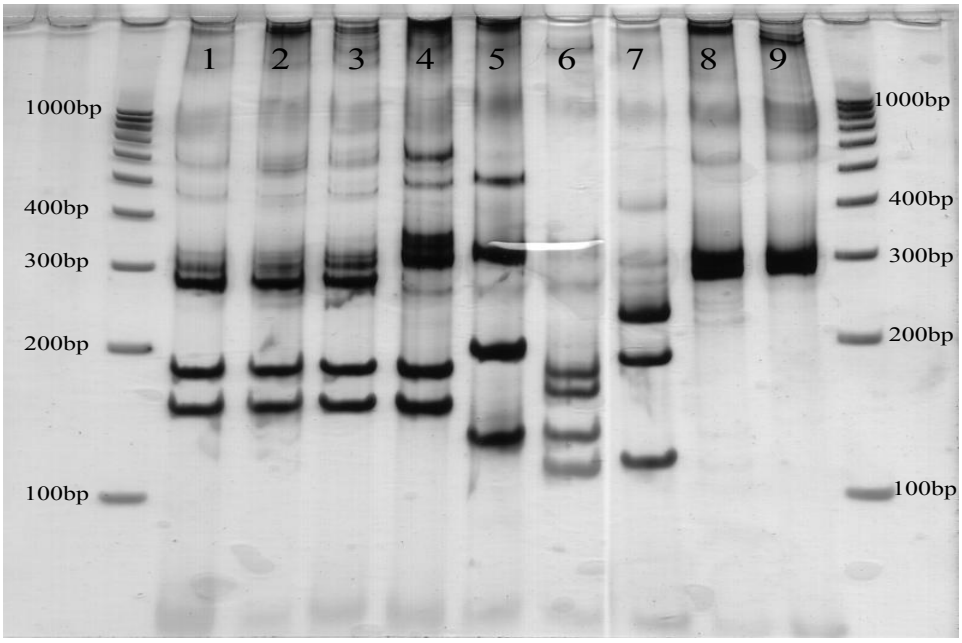


Figure 2.4: Trypanosome species diagnosis using *Msp*I-PCR-RFLP with lanes 1 – 3 *T. congolense* savannah type, 4 *T. congolense* riverine forest, 5 *T. congolense* kilifi, 6 *T. brucei brucei,* 7 *T. vivax*, 8 *T. theileri*, 9 *T. simiae*

2.3.2 Output of the *Dpn*II-PCR-RFLP and whole genome amplification

Out of the 225 *T. congolense*–positive samples, 59 (26%) were successfully amplified using the *Dpn*II-PCR-RFLP. By adding a step of whole genome amplification, an extra 42 samples became positive, reaching a total of 101 (44.9%). The *Dpn*II-PCR-RFLP profiles of the 101 amplified *T. congolense* samples were distributed as follows: 92% presented a resistant genotype, 2% a sensitive genotype and 6% presented a mixed genotype (Figure 2.5).

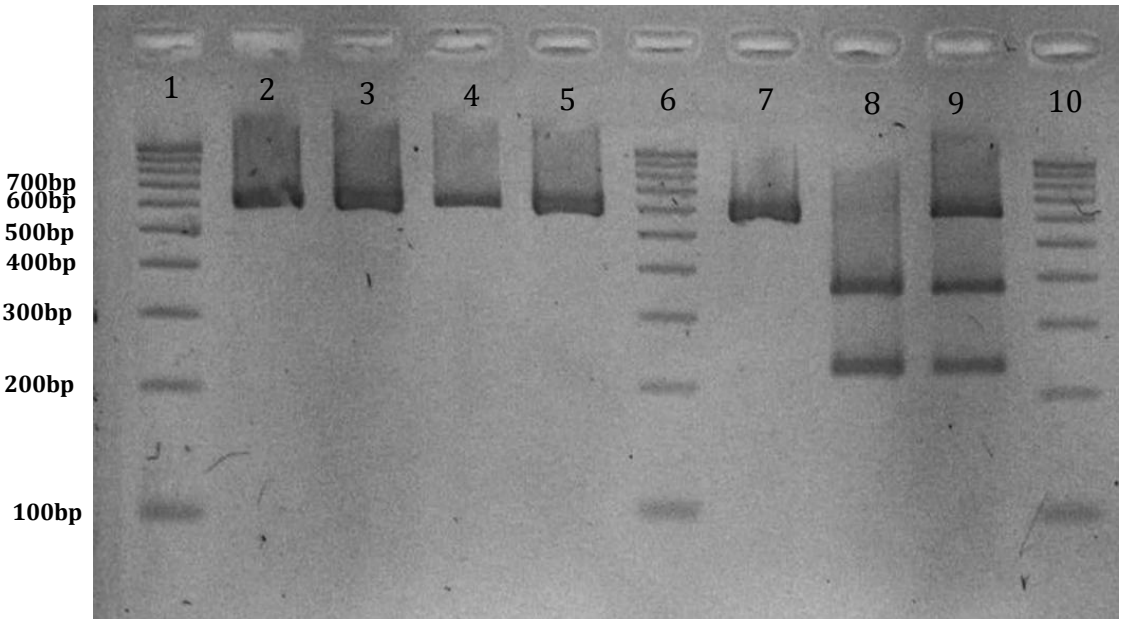
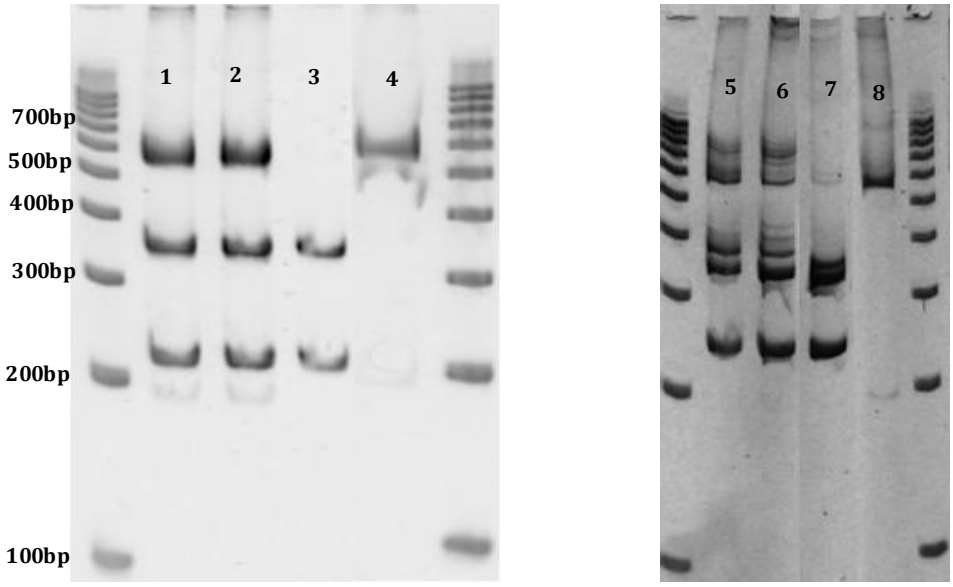


Figure 2.5: Output of the *Dpn*II-PCR-RFLP with lanes 1, 6, and 10 as size markers (100-bp ladder), lanes 2, 3, 4, 5, and 7 as sensitive profiles (one band), lane 8 as resistant profile (two bands), and lane 9 as mixed profile (three bands).

Figure 2.6 illustrates the improved performance of the *Dpn*II-PCR-RFLP compared to the previous *Bcl*I-PCR-RFLP on the same set of samples.



(A) (B)

Figure 2.6: Comparison of *Dpn*II-PCR-RFLP (A) and *Bcl*I-PCR-RFLP (B) profiles of some samples with a mixed profile (1-2), a resistant profile, completely restricted (3), a sensitive profile (4), a mixed profile (5-6), a resistant profile with incomplete restriction (7), a sensitive profile (8).

Digestion of the PCR products was more complete with the *Dpn*II restriction enzyme compared to *Bcl*I (Figure 2.6. lane 3 shows a complete restriction, figure 2.6. lane 7 shows an incomplete restriction). The agarose gel (figure 2.5.) has the advantage to be easier to make and non-toxic when compared to the polyacrylamide gel (figure 2.6.).

Therefore we can state that our objectives to improve the PCR-RFLP for identification of DA-resistance in *T. congolense* were achieved: 1) higher sensitivity as the number of positive PCR's was increased by a factor 1,7 through the use of whole genome amplification, and 2) more complete restriction of the "resistant" amplicons by the use of an alternative restriction enzyme *Dpn*II.

The newly improved PCR-RFLP was then used to assess the current situation of TDR in West Africa in the framework of the RESCAO (Vitouley et al., 2013). A total of 911 samples were collected in suspected treatment failure areas (Murray et al., 1977) of 6 West African countries and analyzed exactly as described above. The obtained results are presented below.

2.3.3. Current situation of DA-resistance in West Africa using molecular tools

Out of the 911 samples collected from 6 West African countries, approximately 21% (194/911) were diagnosed in the *Msp*I-PCR-RFLP as *T. congolense* or mixed *T. congolense* infections. A total of 45% (88/194) of the latter samples were successfully amplified with the PCR Ade2. The *Dpn*II-PCR-RFLP profiles of these samples were distributed as follow: 83% presented a DA-resistant genotype, 2% a DA-sensitive genotype, while 15% presented a mixed-DA genotype (Table 2.1). Recently, such a high level of the DA-resistance genotype was also observed in the Ghibe river basin (Ethiopia) where 94.6% of the samples successfully amplified in the *Dpn*II-PCR-RFLP presented a DA-resistant genotype (Moti et al., 2012).

Although these results suggest an alarming situation, they might not always reflect the real situation in the field as a resistant profile might be classified as sensitive when counterchecked in the mice test at the discriminating dose of 20mg/kg (Delespaux et al., 2006). The molecular technique allows then for the identification of some resistant genotypes that still have sensitive phenotypes in the field. In West Africa for example, when using the block treatment method, the proportion of reported treatment failure to DA is lower with observed prevalences ranging from 8.6% (n=93) in Burkina Faso (Sow et al., 2012) to 42.2% (19/45) in Mali (Mungube et al., 2012).

The molecular method appears thus to be much more sensitive than the mice test and the block treatment technique. Consequently, it can be argued whether or not the use of this molecular test is useful. In other words, does this higher sensitivity constitute an advantage for the establishment of adequate control strategies? What to do with the animals that are infected with “resistant” trypanosomes (harboring the mutation) but that would be cured with a normal dosage of the drug? These issues will be more extensively handled in the general discussion.

Table 2.1: PCR-RFLP outputs of the samples collected in 6 West African countries

Country	Number of field samples received	*Msp*I-PCR-RFLP (*Tc* and mixed *Tc*)	PCR Ade2+	*Dp*nII-PCR-RFLP		
				R	S	M
Benin	25	7	4	4	0	0
Burkina Faso	350	62	28	19	0	9
Côte d'Ivoire	313	100	39	33	2	4
Ghana	20	11	9	9	0	0
Nigeria	149	7	3	3	0	0
Togo	54	7	5	5	0	0
Total (%)	911	194 (21%)	88 (45%)	73 (83%)	2 (2%)	13 (15%)

R, S, and M as *T. congolense* with respectively a resistant, sensitive, and mixed *Dpn*II-PCR-RFLP profile.

2.4. Conclusions

Notwithstanding the suboptimal storage procedure, it can be concluded that blood samples collected and stored on filter papers can be used for detecting the presence of trypanosomes resistant to DA in a trypanosome population. Moreover, losses due to the sub-optimal storage conditions can be compensated for by increasing the sample size. Considering the important logistical and economic advantages of filter papers, their use greatly facilitates the implementation of large scale surveys for TDR in trypanosomosis-affected African countries using molecular diagnostic tools. Furthermore, the use of molecular tools greatly supports the functioning and sustainability of much-needed regional reference laboratories (25€ for species identification and DA resistance detection in *Dpn*II-PCR-RFLP, compared to a test in mice -150€ all included except technician salary and transport - and block treatment which costs 945€ for a survey of treatment failure in a village herd). However, the results of the analysis performed with the improved RFLP-test must be interpreted with caution as some strains presenting a resistant genotype, were detected in the field with a sensitive phenotype. The opposite was never observed. There is need to better understand and characterize the link between the presence of the mutation and the level of drug resistance observed.

Finally, it might be interesting to prospect the development of a similar molecular tool for the diagnosis of DA-resistance in *T. vivax,* as it is the predominant trypanosome species found in West Africa (Kalu et al., 2001; Nakayima et al., 2012; Sow et al., 2012).

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2.5. Reference List

de Almeida, P.J., Ndao, M., Van, M.N., Geerts, S., 1997. Diagnostic evaluation of PCR in goats experimentally infected with *Trypanosoma vivax*. Acta Trop. 66, 45-50.

Delespaux, V., Chitanga, S., Geysen, D., Goethals, A., Van den Bossche, P., Geerts, S., 2006. SSCP analysis of the P2 purine transporter TcoAT1 gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Trop. 100, 96-102.

Eisler, M.C., Brandt, J., Bauer, B., Clausen, P.H., Delespaux, V., Holmes, P.H., Ilemobade, A., Machila, N., Mbwambo, H., McDermott, J., Mehlitz, D., Murilla, G., Ndung'u, J.M., Peregrine, A.S., Sidibe, I., Sinyangwe, L., Geerts, S., 2001. Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. Vet. Parasitol. 97, 171-182.

Eisler, M.C., McDermott, J., Mdachi, R.E., Murilla, G.A., Sinyangwe, L., Mubanga, J., Machila, N., Mbwambo, H., Coleman, P., Clausen, P.H., Bauer, B., Sidibe, I., Geerts, S., Holmes, P.H., Peregrine, A.S., 2000. Rapid method for the assessment of trypanocidal drug resistance in the field. Proceedings of the 9th Symposium of the International Society for veterinary epidemiology and economics, Nairobi, paper 353, 1-3.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171-180.

Kalu, A.U., Oboegbulem, S.I., Uzoukwu, M., 2001. Trypanosomosis in small ruminants maintained by low riverine tsetse population in central Nigeria. Small Rumin. Res. 40, 109-115.

Mamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S., 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. Acta Trop. 106, 115-118.

Moti, Y., Fikru, R., Van Den Abbeele, J., Buscher, P., Van den Bossche, P., Duchateau, L., Delespaux, V., 2012. Ghibe river basin in Ethiopia: present situation of trypanocidal drug resistance in *Trypanosoma congolense* using tests in mice and PCR-RFLP. Vet. Parasitol. 189, 197-203.

Mungube, E.O., Vitouley, H.S., Cudjoe, E.A., Diall, O., Boucoum, Z., Diarra, B., Sanogo, Y., Randolph, T.F., Bauer, B., Zessin, K.H., Clausen, P.H., 2012. Detection of multiple drug-resistant *Trypanosoma congolense* populations in village cattle of south-east Mali. Parasit. Vectors 5, 155.

Murray, M., Murray, P.K., Mcintyre, W.I.M., 1977. Improved parasitological technique for diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 71, 325-326.

Nakayima, J., Nakao, R., Alhassan, A., Sugimoto, C., 2012. Molecular epidemiological studies on animal trypanosomiases in Ghana. Parasit. Vectors. 5, 217.

Sow, A., Sidibe, I., Bengaly, Z., Marcotty, T., Sere, M., Diallo, A., Vitouley, H.S., Nebie, R.L., Ouedraogo, M., Akoda, G.K., Van den Bossche, P., Van Den Abbeele, J., De, D.R., Delespaux, V., 2012. Field detection of resistance to isometamidium chloride and diminazene aceturate in *Trypanosoma vivax* from the region of the Boucle du Mouhoun in Burkina Faso. Vet. Parasitol. 187, 105-111.

Stephen, L.E., 1986. Trypanosomiasis: a veterinary perspective. Pergamon Press, UK. (ISBN 0-08-032017-1). 551 p.

Vitouley, H.S., Bengaly, Z., Adakal, H., Sidibe, I., Van Den Abbeele, J., Delespaux, V., 2013. Réseau d'EpidémioSurveillance de la Chimiorésistance aux trypanocides et aux acaricides en Afrique de l'Ouest (RESCAO). Tropicultura 31, 205-212.

Chapter 3

Prospecting the development of a molecular diagnostic tool for *Trypanosoma vivax* resistance to diminazene aceturate.

3.1. Introduction

T. vivax infections are widespread in sub-Saharan Africa. It is the predominant parasite encountered in small wild vertebrates in South Cameroon (Njiokou et al., 2004), in small ruminants in Nigeria (Kalu et al., 2001) and in pigs and cattle in Ghana (Nakayima et al., 2012). The control of the trypanosome infections in the animal host still heavily relies on the use of three trypanocidal drugs i.e. ISM, DA and homidium salts.

The molecular tools that were recently developed permit the rapid diagnosis of TDR in *T. congolense* and in *T. brucei.* Those tools were used in the framework of large-scale epidemiological surveys in different countries of sub-Saharan Africa (de Koning et al., 2004; Delespaux et al., 2006; Vitouley et al., 2011). Unfortunately, up to now, for *T. vivax,* the page remains blank in this domain. Therefore, the objective of the work reported in this chapter was to explore the possibility of the development of a similar technique for *T. vivax*. The study consisted of the systematic screening of the genes of *T. vivax* coding for nucleoside transporters. Up to now, no functional analysis has been performed on the nine nucleoside transporters described in the genomic databases available for *T. vivax*. At the moment of the study, it was not possible to discriminate the P1 from the P2 type transporters. Even if in *T. congolense* a P1-type purine transporter (*TcoNT10*) was identified as a genetic marker associated with DA resistance, a test based on a mutation in a P2-type purine transporter identified as associated with the resistant phenotypes would bring some evidence in the scientific community (Delespaux et al., 2006; Mäser et al., 2003). As a first approach in our exploration, we decided to go for a systematic screening of the available nine *T. vivax* open reading frames that are predicted to code for putative nucleoside transporters (Jackson et al., 2013). Subsequently, SSCP and sequencing analysis were performed, on the four genes of *T. vivax* clading with the *T. brucei* genes, to detect point mutations that could possibly be linked to decreases in sensitivity to DA.

3.2. Materials and methods

3.2.1. Materials used

Five *T. vivax* samples were used for the SSCP analysis and sequencing, in which one (ILRAD700) was the sensitive reference strain (Andrikaye, 2008) and four were field isolates characterized for their sensitivity/resistance to DA in goats (Vitouley et al., 2012; Delespaux, pers. Comm.). Table 3.1 summarizes the details about the isolates.

3.2.2. Methods

3.2.2.1. DNA extraction

Four of the five the *T. vivax* samples used in this study were buffy coats stored in 0.5 ml Eppendorf tubes containing 30 µl of sterile distilled water. These buffy coats were homogenized after which 100µl of 1% aqueous solution of Chelex® (BIORAD) was added to the tubes and vortexed slowly. The tubes were then placed in a heating block for 1h at 56°C and then 30min at 95°C. Finally, the tubes were centrifuged for 2 min at 15 000g and the supernatants (containing the parasite DNA) were collected in new Eppendorf tubes. The DNA extracts were stored at -20°C for subsequent PCR analysis (Solano et al., 1999). For the reference sample, i.e. the mouse adapted ILRAD700, DNA extraction from mouse blood was performed using the QIAamp DNA Blood Mini Kit (Qiagen).

3.2.2.2. PCR-RFLP for species diagnosis

In order to confirm the trypanosome species, a first PCR based on the small ribosomal sub-unit 18S was performed on the DNA extracts using 18STnF2/18STnR3 and 18STnF2/18STnR2 as first and second round primers, respectively. Subsequently, the PCR products were digested with the *Msp*I restriction enzyme (Geysen et al., 2003; Vitouley et al., 2011).

Table 3.1: *T. vivax* samples used in the SSCP and cloning studies

DNA code	Sensitivity to DA	Host	Village	Country	Provided by	Conservation
DE51Tv/Ch.D, 03/04/09 (H65)	resistant	Goat	Dèrè	Burkina Faso	CIRDES	Buffy coat
MOU18Tv/Ch.A, 11/04/09 (H89)	resistant	Goat	Mou	Burkina Faso	CIRDES	Buffy coat
DEB53Tv/F, 09/04/09 (H988)	sensitive	Goat	Débé	Burkina Faso	CIRDES	Buffy coat
GH12Tv/Ch.A62, 29/05/08 (H1008)	sensitive	Goat	Kunchugu	Ghana	CIRDES	Buffy coat
ILRAD700	sensitive	Mouse	-	Belgium	ITM	Stock solution*

* For the reference sample, the mouse adapted ILRAD700, DNA extraction from mouse blood was performed using the QIAamp DNA Blood Mini Kit (Qiagen).

3.2.2.3. Single Strand Conformation Polymorphism (SSCP) analysis

Sets of primers (Table 3.2) were designed using the Primer3 (Rozen and Skaletsy, 2000) computer program (http://frodo.wi.mit.edu/). PCR and subsequent SSCP analysis were then performed on the 1395bp, 1419bp, 1413bp, 1122bp nucleoside transporter genes with their respective predicted sequences Chr6Tviv400 (TvY486_0043680), Chr2Tviv585 (TvY486_0202110), ChrUnknownTviv1924 (TvY486_1112030), ChrUnknownTviv275 (TvY486_0014570) (GeneDB-Welcome Trust Sanger Institute, http://www.genedb.org). To detect sequence differences (point mutations) that could possibly be linked to a decreased sensitivity to DA, we firstly used Single Strand Conformation Polymorphism (SSCP) on the 4 genes of *T. vivax* clading with the purine transporters of *T. brucei* (Figure 3.1). Subsequently, sequencing was then used to compare the open reading frame sequences.

Standard PCR amplifications were carried out (40 cycles) as described in the second chapter, except for the *T. vivax* specific primer-sets that were used (see Table 3.2). Electrophoresis was performed in a 1% agarose gel for 20min (100V) and stained with ethidium bromide for 30min. Next, the obtained PCR products were digested using two restriction enzymes per amplicon. The two restriction sites were located at least 50bp from each other to ensure that - at least on one of the amplicons - the restriction did not happen too close of a potential mutation rendering its detection impossible. To allow optimal SSCP analysis, restriction enzymes were chosen to cut the amplicons in fragments of an adequate size i.e. between 100 and 300bp. Digestions were then performed with the restriction enzymes mentioned in table 3.2 in the appropriate NEBuffer (New England BioLabs) according to the manufacturer's specifications, using 10 units µg DNA (0.6 U/µl PCR product) on 6 µl of amplified DNA in 15 µl total volume. The reaction was incubated overnight at the specified temperature. The electrophoresis and staining for the SSCP analysis was performed as follow: 8 µl of D2 buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was mixed with 4 µl of digested PCR product. The mixture was then heated for 5 min at 95°C, cooled on ice and left for at least 10 min at -18°C. Thereafter, 6µl of this mixture was loaded in the wells of a polyacrylamide gel (PAGE gel) and the electrophoresis was run at 100V for 2h30 min.

Table 3.2: *T. vivax* nucleoside transporters explored in the SSCP, cloning and sequencing analysis

Amplicons	Size (bp)	Restriction enzymes	Forward primer	Reverse primer
TvY486_0043680 (1)	625	*Ags*I-*BamH*I	AGGTGCGCTCTCAACTGAAT	CCAGAAAGTGGGAGTCCAAA
TvY486_0043680 (2)	606	*Aar*I-*PflF*I	CCGGTGCAAAGACGTTATTC	AGAAGAGGAGCGTTGAGCAG
TvY486_0043680 (3)	703	*RleA*I-*Eae*I	TGATCAGGTCGAAAACACCA	TTCATTTTGCTTTGCACCAC
TvY486_0202110 (1)	596	*BamH*I-*Cac8*I	GCAAACATGATTCTTTCTCTTTGAT	ATAATATACCGGTGATGACTCCAGA
TvY486_0202110 (2)	596	*BseR*I-*Hin4*I	CAATGGAATTGAAAAATCTCTCTGT	GGAAAAACAAAACTGTTGTGAAATA
TvY486_0202110 (3)	536	*Drd*IV-*BseR*I	CTATTTCACAACAGTTTTGTTTTTCC	ACAAACACTTATTTACCACACAGGTT
TvY486_1112030 (1)	517	*BamH*I-*Rsa*I	CCACCGGCTCTATATATTCCTTATAC	GCGATGGAAAAATATATGTATGACTG
TvY486_1112030 (2)	642	*Dpn*II-*Rsa*I	TCCATCAGTCATACATATATTTTTCCA	CATTACCTTCTATTATACGACGTTGG
TvY486_1112030 (3)	608	*Alw*I-*Mbo*II	CGTCTACTTCTCAACATTTGTGTTTT	TCACGAATTGATGAATAACTTCTTTC
TvY486_0014570 (1)	656	*BamH*I-*SfaN*I	TCCTGTCTTCACACTATTAGACAAGC	GAGAAGTATATGTACGACTGGGTCAA
TvY486_0014570 (2)	631	*BstN*I-*Alw*I	CTCTTGACCCAGTCGTACATATACTT	GTTGTCCTTTATCACCTTCTGTTGAC
TvY486_1103740 (1)	585	-	TGTTGTCCAGCTGCTTTCCTA	TTCTCCATCGACCCCTTGAT
TvY486_1103740 (2)	565	-	ACATATGGAGGCCACCACTTAC	ATCACCAAAGTTGTAGCACAGAATC
TvY486_1103740 (3)	501	-	TTCCTTCTTTGTCACGCTCTTTAT	CAGTAAACGCTGCTACGTCTTAAA
TvY486_0041960 (1)	586	-	TGCTGAAGTGACATCGAAGG	ACGACACCAGCGTGTAAGTG
TvY486_0041960 (2)	596	-	CACTTACACGCTGGTGTCGT	AGGGCCATATGCACTTGAAC
TvY486_0041960 (3)	415	-	TCTTGTCCTTCCCATTGACC	AAGGTACTGTTAGGGCAAAGC
TvY486_0011610 (1)	631	-	CAAATATGCTCCTGGGCTTCT	ATGACAAGTGAGGCGGCTAT
TvY486_0011610 (2)	439	-	CACTTACACGCTGGTGTCGT	CGTTCTCCTGTGTCAGGTGT
TvY486_1103750 (1)	595	-	TGCTGAAGTGACATCGAAGG	GATAACGGCGCAGGTTCG
TvY486_1103760 (1)*	666	-	AGGGGGATCCTCTAGAGTCG	TTCATCATGCGGTACTCAGC
TvY486_1103760 (2)*	688	-	CGCTACATTTCCTACGCACA	ATGCCAATGGGCAGTCTTAG

TvY486_…(x) as the x amplicon of the gene TvY486_…
TvY486_… without indication of restriction enzymes as the additional *T. vivax* nucleoside transporters genes explored only in the sequencing analysis (i.e. TvY486_1103740, TvY486_0041960, TvY486_0011610, TvY486_1103750, TvY486_1103760)
* Gene fragment, no complete open reading frame

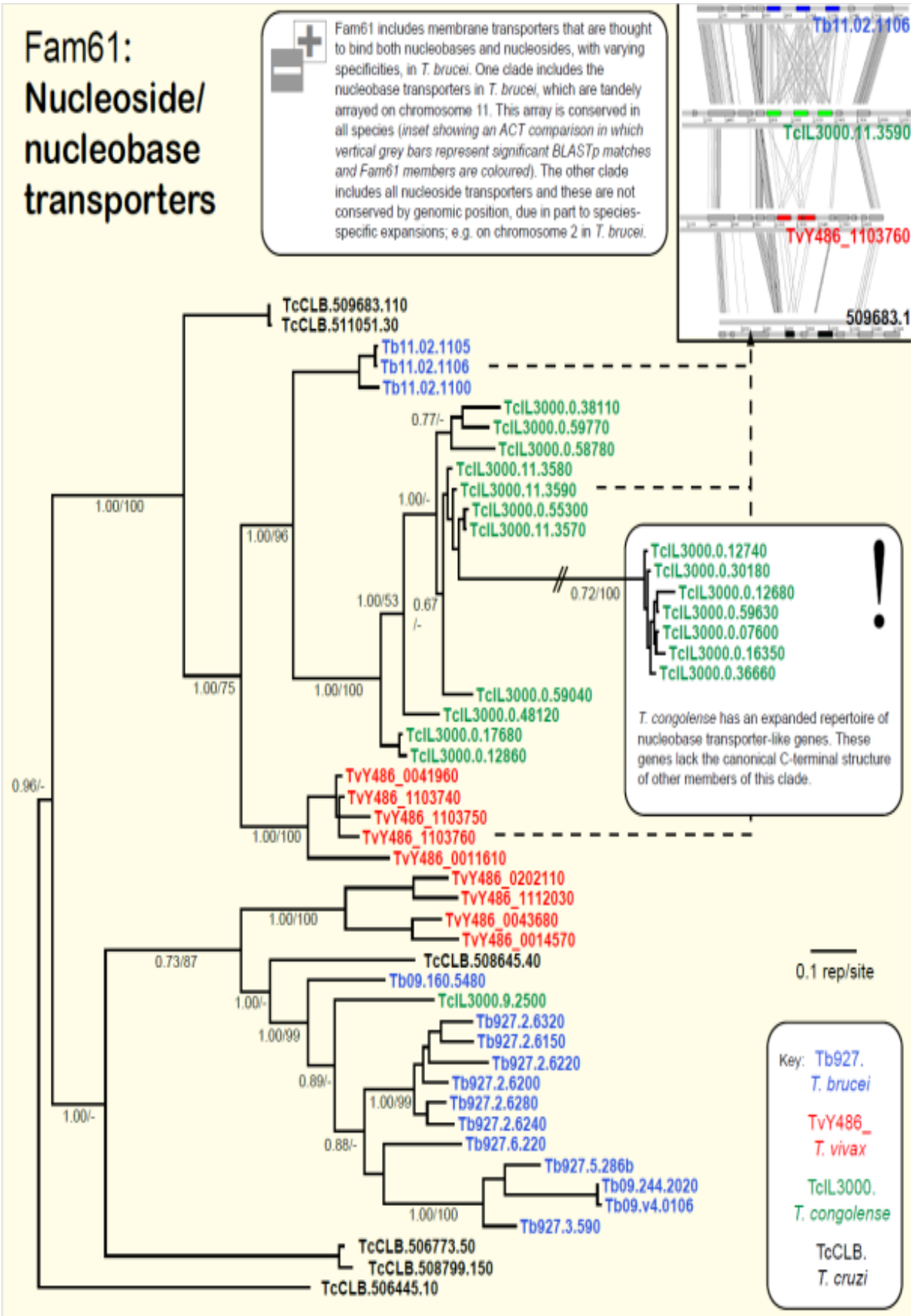


Figure 3.1: The phylogenetic tree of the nucleoside transporters in *T. congolense*, *T. brucei* and *T. vivax* (Jackson et al., 2013). High resolution picture is available at: ftp://ftp.sanger.ac.uk/pub/pathogens/Trypanosoma/surface_phylome/61/Supplementary_figure.pdf

3.2.2.4. Cloning and sequencing

The nine nucleoside transporters genes described in *T. vivax* (Figure 3.1) (Jackson et al., 2013) were explored by amplification and sequencing of two DA-resistant and two DA-sensitive samples (Table 3.1). ILRAD 700 was used as a *T. vivax* reference sensitive strain (Andrikaye, 2008). These samples were amplified as described above for the amplicons of the SSCP analysis except of the additional use of 5 new primers (see Table 3.2) for the transporters cladding with the *T. congolense* nucleoside transporters (Figure 3.1). For the sequencing of the targeting genes, the PCR products were cloned using the Topo-cloning® kit (Invitrogen, Carlsbad, CA, USA), exactly as described by the manufacturer. The recombinant plasmids containing the desired inserts were purified and then sequenced (one clone per isolate for each PCR) using the Model 377-XL Sequencer (PE-Applied Biosystems, Eurogentec®, Belgium). Afterwards, the obtained fragments were aligned using ClustalX 2.0.12 software in order to detect any conserved polymorphism between sensitive and resistant strains.

3.3. Results and discussion

3.3.1. Species diagnosis

All the five samples analyzed in 18S-PCR-RFLP using restriction enzyme *Msp*I were confirmed as *T. vivax* (see Figure 3.2).

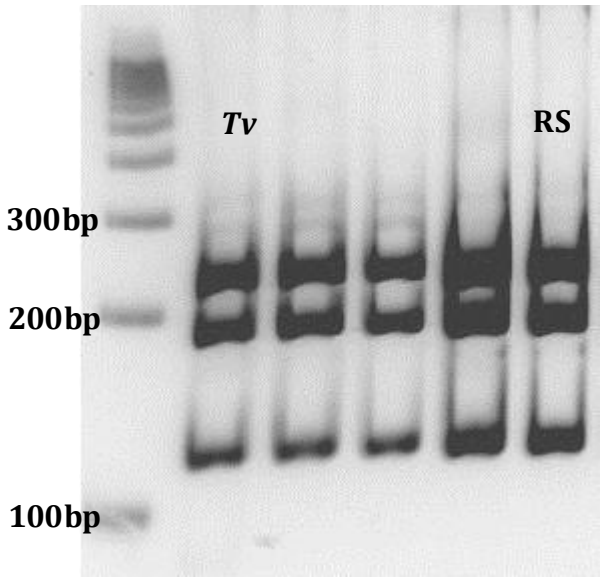
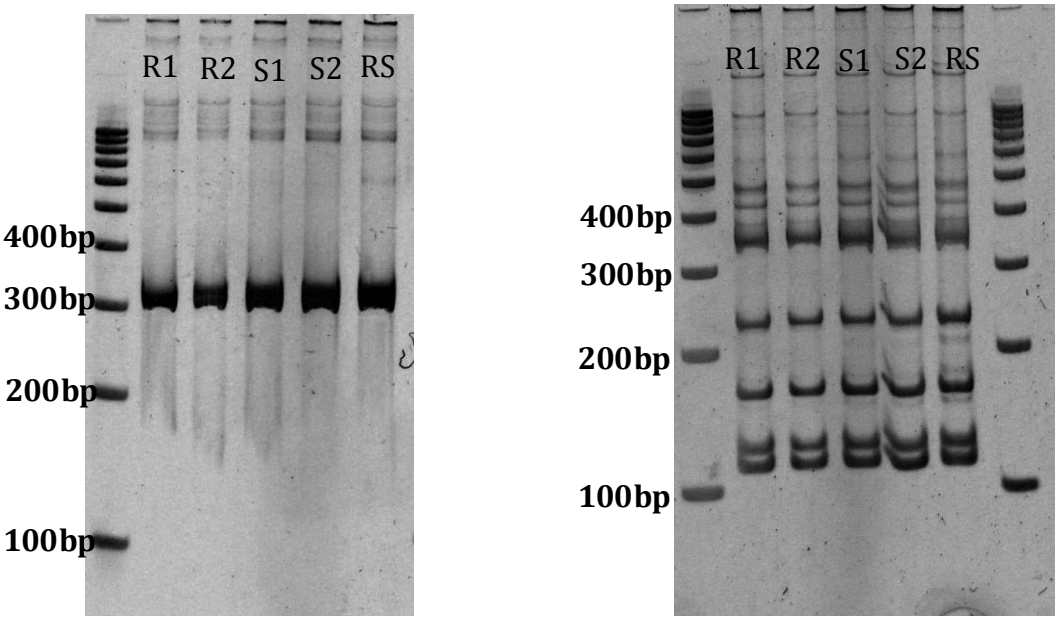


Figure 3.2: PCR-RFLP-*Msp*I profile of *T. vivax* infections
Tv: *T. vivax*; RS for the Reference Sensitive sample i.e. *T.vivax* ILRAD700

3.3.2. Determination of polymorphism related to diminazene aceturate resistance in *T. vivax*

When considering both techniques (SSCP and sequencing), the results showed the conserved character of the explored genes and did not reveal any polymorphism related to DA resistance. Some polymorphism was observed but was not linked to the sensitivity/resistance phenotypes. When considering for example the nucleoside transporter TvY486_1112030, the results of SSCP performed on the first amplicon using sensitive and resistant *T. vivax* strains (Table 3.1) are shown in the figure 3.3 below.



Gel A (*BamH*I) Gel B (*Rsa*I)

Figure 3.3: SSCP analysis performed on the first amplicon of TvY486_1112030 using 5 *T*. vivax samples with *BamH*I and *Rsa*I as restriction enzymes.
R for resistant samples (R1: H65, R2: H89); S (S1: H988, S2: H1008) for sensitive samples and RS for the reference sensitive sample.

As can be observed in the above figure 3.3, sensitive and resistant strains have the same SSCP profile for the two restriction enzymes used. Concerning the sequence analysis of this amplicon, the obtained results after alignment with Clustal X 2.0.12 are shown hereafter. No conserved polymorphism was observed between sensitive and resistance strains as we observed previously in SSCP analysis of the same amplicon of TvY486_1112030.

```
>TvY486_1112030F1       CCACCGGCTCTATATATTCCTTATAC
>TvY486_1112030R1       GCGATGGAAAAATATATGTATGACTG

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           CCACCGGCTCTATATATTCCTTATACAGCTACTCCACAGAATTTTACAAAATGGCTCAGG
H89_resistant           CCACCGGCTCTATATATTCCTTATACAGCTACTCCACAGAATTTTACAAAATGGCTCAGG
H988_sensitive          CCACCGGCTCTATATATTCCTTATACAGCTACTCCACAGAATTTTACAAAATGGCTCAGG
H1008_sensitive         CCACCGGCTCTATATATTCCTTATACAGCTACTCCACAGAATTTTACAAAATGGCTCAGG
ILRAD700_sensitive      CCACCGGCTCTATATATTCCTTATACAGCTACTCCACAGAATTTTACAAAATGGCTCAGG
                        ************************************************************

H65_resistant           GCGACCCGAATGCCACAACCAGCGACCCTGACTTCTGGAAGAATATCTACACGTATTACA
H89_resistant           GCGACCCGAATGCCACAACCAGCGACCCTGACTTCTGGAAGAATATCTACACGTATTATA
H988_sensitive          GCGACCCGAATGCCACAACCAGCGACCCTGACTTCTGGAAGAATATCTACACGTATTATA
H1008_sensitive         GCGACCCGAATGCCACAACCAGCGACCCTGACTTCTGGAAGAATATCTACACGTATTATA
ILRAD700_sensitive      GCGACCCGAATGCCACAACCAGCGACCCTGACTTCTGGAAGAATATCTACACGTATTATA
                        ************************************************************ *

H65_resistant           ACGTGGTTATCTTTTCAACAGAAATCGTTTTTGAATTGTTCATGCTGACGTCGCTTGGCA
H89_resistant           ACGTGGTTATCTTTTCAACAGAAATCGTTTTTGAATTGTTCATGCTGACGTCGCTTGGCA
H988_sensitive          ACGTGGTTATCTTTTCAACAGAAATCGTTTTTGAATTGTTCATGCTGACGTCGCTTGGCA
H1008_sensitive         ACGTGGTTATCTTTTCAACAGAAATCGTTTTTGAATTGTTCATGCTGACGTCGCTTGGCA
ILRAD700_sensitive      ACGTGGTTATCTTTTCAACAGAAATCGTTTTTGAATTGTTCATGCTGACGTCGCTTGGCA
                        ************************************************************

H65_resistant           GAAGGATCCCATTGCGACTGAGGTTGGCGCTAGGCTTCTTCCTTTCGCTTGCACAACTCC
H89_resistant           GAAGGATCCCATTGCGACTGAGGTTGGCGCTAGGCTTCTTCCTTTCGCTTGCACAACTCC
H988_sensitive          GAAGGATCCCATTGCGACTGAGGTTGGCGCTAGGCTTCTTCCTTTCGCTTGCACAACTCC
H1008_sensitive         GAAGGATCCCATTGCGACTGAGGTTGGCGCTAGGCTTCTTCCTTTCGCTTGCACAACTCC
ILRAD700_sensitive      GAAGGATCCCATTGCGACTGAGGTTGGCGCTAGGCTTCTTCCTTTCGCTTGCACAACTCC
                        ************************************************************

H65_resistant           TTTCGTACATGATGGTCACCACATTCCATACAAGCGAATCCGGCGCAAAGTGTATATTTT
H89_resistant           TTTCGTGCATGATGGTCACCACATTCCATACAAGCGAATCCGGCGCAAAGTGTATATTTT
H988_sensitive          TTTCGTACATGATGGTCACCACATTCCATACAAGCGAATCCGGCGCAAAGTGTATATTTT
H1008_sensitive         TTTCGTACATGATGGTCACCACATTCCATACAAGCGAATCCGGCGCAAAGTGTATATTTT
ILRAD700_sensitive      TTTCGTACATGATGGTCACCACATTCCATACAAGCGAATCCGGCGCAAAGTGTATATTTT
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H65_resistant           TGCTTTTTGGCGTTGTCAATGGCGTGGCAAAGACTTTCTGTGCGTCCAGCACTGTGGCAC
H89_resistant           TGCTTTTTGGCGTTGTCAATGGCGTGGCAGAGACTTTCTGTGCGTCCAGCACTGTGGCAC
H988_sensitive          TGCTTTTTGGCGTTGTCAATGGCGTGGCAAAGACTTTCTGTGCGTCCAGCACTGTGGCAC
H1008_sensitive         TGCTTTTTGGCGTTGTCAATGGCGTGGCAAAGACTTTCTGTGCGTCCAGCACTGTGGCAC
ILRAD700_sensitive      TGCTTTTTGGCGTTGTCAATGGCGTGGCAAAGACTTTCTGTGCGTCCAGCACTGTGGCAC
                        ************************** ******************************

H65_resistant           TTGCAGGTCCATTTCCTACAAAAATTTTTAGTGCGTATGTCCTTGGAATCCCATTTGCTG
H89_resistant           TTGCAGGTCCATTTCCTACAAAAATTTTTAGTGCGTATGTCCTTGGAATCCCATTTGCTG
H988_sensitive          TTGCAGGTCCATTTCCTACAAAAATTTTTAGTGCGTATGTCCTTGGAATCCCATTTGCTG
H1008_sensitive         TTGCAGGTCCATTTCCTACAAAAATTTTTAGTGCGTATGTCCTTGGAATCCCATTTGCTG
ILRAD700_sensitive      TTGCAGGTCCATTTCCTACAAAAATTTTTAGTGCGTATGTCCTTGGAATCCCATTTGCTG
                        ************************************************************

H65_resistant           GAGTAATCGCTGGCGTGCTATCGGTAACAATCAAGGCGTCGATGAATGGTGACTTTCACG
H89_resistant           GAGTAATCGCTGGCGTGCTATCGGTAACAATCAAGGCGTCGATGAATGGTGACTTTCACG
H988_sensitive          GAGTAATCGCTGGCGTGCTATCGGTAACAATCAAGGCGTCGATGAATGGTGACTTTCACG
H1008_sensitive         GAGTAATCGCTGGCGTGCTATCGGTAACAATCAAGGCGTCGATGAATGGTGACTTTCACG
ILRAD700_sensitive      GAGTAATCGCTGGCGTGCTATCGGTAACAATCAAGGCGTCGATGAATGGTGACTTTCACG
                        ************************************************************

H65_resistant           GCTTGCTCCATCAGTCATACATATATTTTTCCATCGC
H89_resistant           GCTTGCTCCATCAGTCATACATATATTTTTCCATCGC
H988_sensitive          GCTTGCTCCATCAGTCATACATATATTTTTCCATCGC
H1008_sensitive         GCTTGCTCCATCAGTCATACATATATTTTTCCATCGC
ILRAD700_sensitive      GCTTGCTCCATCAGTCATACATATATTTTTCCATCGC
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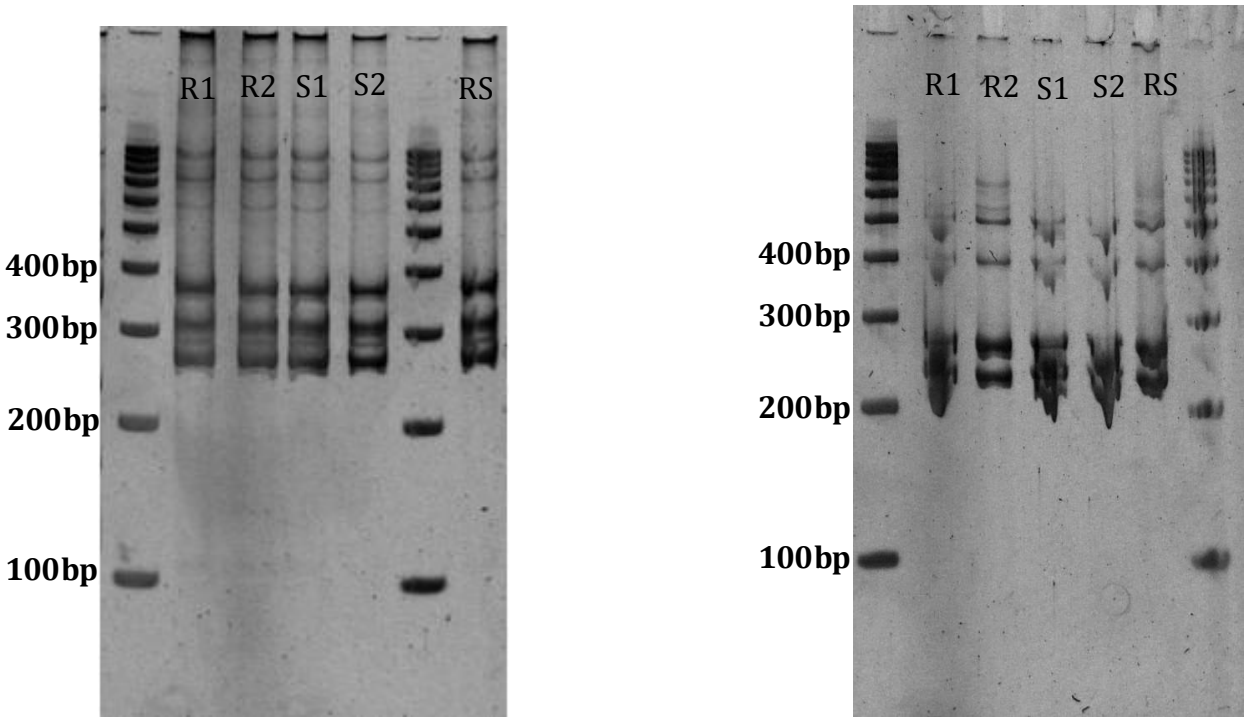
A similar result was observed with the second amplicon of TvY486_1112030 in SSCP analysis as is shown in Fig. 3.4.



Gel C (*Dpn*II) Gel D (*Rsa*I)

Figure 3.4: SSCP analysis performed on the second amplicon of TvY486_1112030 using 5 *T. vivax samples* with *Dpn*II and *Rsa*I as restriction enzymes.
R for resistant samples (R1: H65, R2: H89); S (S1: H988, S2: H1008) and RS for the reference sensitive sample.

For the sequencing results of the second amplicon of TvY486_1112030, some polymorphism was observed, but this was not linked to the sensitivity / resistance phenotypes (see hereafter).

```
>TvY486_1112030F2       TCCATCAGTCATACATATATTTTTCCA
>TvY486_1112030R2       CATTACCTTCTATTATACGACGTTGG

CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           TCCATCAGTCATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCC
H89_resistant           TCCATCAGTCATACATATATTTTTCCATCGCTATGGTTTTTCACTCGGTGACCTGCGTCC
H988_sensitive          TCCATCAGTCATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCC
H1008_sensitive         TCCATCAGTCATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCC
ILRAD700_sensitive      TCCATCAGTCATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCC
                        ***************************************** ****************

H65_resistant           TTTTGTATTTGCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTTGGTACG
H89_resistant           TTTTGTATTTGCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAGTTTAGGTACG
H988_sensitive          TTTTGTATTTGCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACG
H1008_sensitive         TTTTGTATTTGCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACG
ILRAD700_sensitive      TTTTGTATTTGCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACG
                        ********************************************** *** ******

H65_resistant           CGGCGAGGGGTAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTG
H89_resistant           CGGCGAGGGGTAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTG
H988_sensitive          CGGCGAGGGGTAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTG
H1008_sensitive         CGGCGAGGGGTAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTG
ILRAD700_sensitive      CGGCGAGGGGTAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTG
                        ************************************************************

H65_resistant           CACCTGATTCGCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGT
H89_resistant           CACCTGATTCGCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGT
H988_sensitive          CACCTGATTCGCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGT
H1008_sensitive         CACCTGATTCGCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGT
ILRAD700_sensitive      CACCTGATTCGCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGT
                        ************************************************************

H65_resistant           ACAACACTGCAAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTG
H89_resistant           ACAACACTGCAAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTG
H988_sensitive          ACAACACTGCAAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTG
H1008_sensitive         ACAACACTGCAAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTG
ILRAD700_sensitive      ACAACACTGCAAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTG
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H65_resistant          ACCAGGTGGAGAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCA
H89_resistant          ACCAGGTGGAGAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCA
H988_sensitive         ACCAGGTGGAGAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCA
H1008_sensitive        ACCAGGTGGAGAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCA
ILRAD700_sensitive     ACCAGGTGGAGAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCA
                       ************************************************************

H65_resistant          TCAAGCGCATATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGT
H89_resistant          TCAAGCGCATATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGT
H988_sensitive         TCAAGCGCATATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGT
H1008_sensitive        TCAAGCGCATATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGT
ILRAD700_sensitive     TCAAGCGCATATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGT
                       ************************************************************

H65_resistant          TTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTGGAATCACTGGTACGGCACTG
H89_resistant          TTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTGGAATCACTGGTACGGCACTG
H988_sensitive         TTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTGGAATCACTGGTACGGCACTG
H1008_sensitive        TTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTGGAATCACTGGTACGGCACTG
ILRAD700_sensitive     TTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTGGAATCACTGGTACGGCACTG
                       ************************************************************

H65_resistant          CGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTGTATGTTTCTTCAGTTCAAGA
H89_resistant          CGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTGTATGTTTCTTCAGTTCAAGA
H988_sensitive         CGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTGTATGTTTCTTCAGTTCAAGA
H1008_sensitive        CGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTGTATGTTTCTTCAGTTCAAGA
ILRAD700_sensitive     CGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTGTATGTTTCTTCAGTTCAAGA
                       ************************************************************

H65_resistant          GAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATTCGCTCGCCTACTGATTGCAG
H89_resistant          GAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATTCGCTCGCCTACTGATTGCAG
H988_sensitive         GAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATTCGCTCGCCTACTGATTGCAG
H1008_sensitive        GAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATTCGCTCGCCTACTGATTGCAG
ILRAD700_sensitive     GAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATTCGCTCGCCTACTGATTGCAG
                       ************************************************************

H65_resistant          TACCGCTTTTCCTTTGCCAACGTCGTATAATAGAAGGTAATG
H89_resistant          TACCGCTTTTCCTTTGCCAACGTCGTATAATAGAAGGTAATG
H988_sensitive         TACCGCTTTTCCTTTGCCAACGTCGTATAATAGAAGGTAATG
H1008_sensitive        TACCGCTTTTCCTTTGCCAACGTCGTATAATAGAAGGTAATG
ILRAD700_sensitive     TACCGCTTTTCCTTTGCCAACGTCGTATAATAGAAGGTAATG
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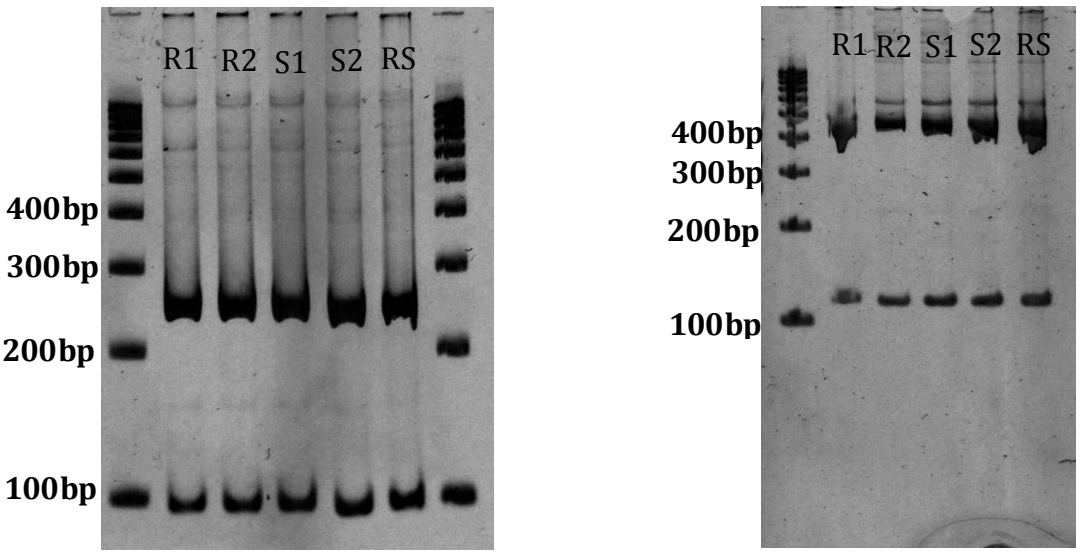
Concerning the third amplicon of TvY486_1112030, resistant and sensitive *T. vivax* strains present the same SSCP profile as is observed in the Figure 3.5.



Gel E (*Al*I) Gel F (*Mbo*II)

Figure 3.5: SSCP analysis performed on the third amplicon of TvY486_1112030 using 5 *T. vivax* samples with *Al*I and *Mbo*II as restriction enzymes
R for resistant samples (R1: H65, R2: H89); S (S1: H988, S2: H1008) and RS for the reference sensitive sample.

For the sequencing of the third amplicon of TvY486_1112030, some polymorphism was observed but was not linked to the sensitivity / resistance phenotypes, as we can see hereafter.

```
>TvY486_1112030F3       CGTCTACTTCTCAACATTTGTGTTTT
>TvY486_1112030R3       TCACGAATTGATGAATAACTTCTTTC

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           CGTCTACTTCTCAACATTTGTGTTTTTCCCTGGAGTTTTTACCTCGGTGGATTACAAGGG
H89_resistant           CGTCTACTTCTCAACATTTGTGTTTTTCCCTGGAGTTTTTACCTCGGTGGATTACAAGGG
H988_sensitive          CGTCTACTTCTCAACATTTGTGTTTTTCCCTGGAGTTTTTACCTCGGTGGATTACAAGGG
H1008_sensitive         CGTCTACTTCTCAACATTTGTGTTTTTCCCTGGAGTTTTTACCTCGGTGGATTACAAGGG
ILRAD700_sensitive      CGTCTACTTCTCAACATTTGTGTTTTTCCCTGGAGTTTTTACCTCGGTGGATTACAAGGG
                        ************************************************************

H65_resistant           TTGGAATCACTGGTACGGCACTATTGTAATAGTTCTGTTCAATTTCGGGGATTTTTTTTC
H89_resistant           TTGGAATCACTGGTACGGCACTATTGTAATAGTTCTGTTCAATTTCGGGGATTTTTTTTC
H988_sensitive          TTGGAATCACTGGTACGGCACTATTGTAATAGTTCTGTTCAATTTCGGGGATTTTTTTTC
H1008_sensitive         TTGGAATCACTGGTACGGCACTATTGTAATAGTTCTGTTCAATTTCGGGGATTTTTTTTC
ILRAD700_sensitive      TTGGAATCACTGGTACGGCACTATTGTAATAGTTCTGTTCAATTTCGGGGATTTTTTTTC
                        ************************************************************

H65_resistant           GGGTGTTCTGCTTCAATTCAAAAAGTTTCACCTATCACCGAAGGCAGCGATCATAGGGTC
H89_resistant           GGGTGTTCTGCTTCAATTCAAAAAGTTTCACCTATCACCGAAGGCAGTGATCATAGGGTC
H988_sensitive          GGGTGTTCTGCTTCAATTCAAAAAGTTTCACCTATCACCGAAGGCAGTGATCATAGGGTC
H1008_sensitive         GGGTGTTCTGCTTCAATTCAAAAAGTTTCACCTATCACCGAAGGCAGTGATCATAGGGTC
ILRAD700_sensitive      GGGTGTTCTGCTTCAATTCAAAAAGTTTCACCTATCACCGAAGGCAGTGATCATAGGGTC
                        ********************************************** ************

H65_resistant           GTTTGGCCGTCTACTGATCGCTGTCCCGCTAGTGGTATGCCAACGTCGTATAATAGAAGG
H89_resistant           GTTTGGCCGTCTACTGATCGCTGTCCCGCTAGTGGTATGCCAACGTCGTATAATAGAAGG
H988_sensitive          GTTTGGCCGTCTACTGATCGCTGTCCCGCTAGTGGTATGCCAACGTCGTATAATAGAAGG
H1008_sensitive         GTTTGGCCGTCTACTGATCGCTGTCCCGCTAGTGGTATGCCAACGTCGTATAATAGAAGG
ILRAD700_sensitive      GTTTGGCCGTCTACTGATCGCTGTCCCGCTAGTGGTATGCCAACGTCGTATAATAGAAGG
                        ************************************************************

H65_resistant           TAATGCCGCCAAAGCGTTGTCCTGTGTTCTCTCACTCCTCTGGGGTATCACAAACGGTTT
H89_resistant           TAATGCCGCCAAAGCGTTGTCCTGTGTTCTCTCACTCCTCTGGGGTATCACAAACGGTTT
H988_sensitive          TAATGCCGCCAAAGCGTTGTCCTGTGTTCTCTCACTCCTCTGGGGTATCACAAACGGTTT
H1008_sensitive         TAATGCCGCCAAAGCGTTGTCCTGTGTTCTCTCACTCCTCTGGGGTATCACAAACGGTTT
ILRAD700_sensitive      TAATGCCGCCAAAGCGTTGTCCTGTGTTCTCTCACTCCTCTGGGGTATCACAAACGGTTT
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H65_resistant          TTGCGGCGGCATGATGCTTATTTACGGATCGAGGACTGCATCATTGACAACGGCAGGCCA
H89_resistant          TTGCGGCGGCATGATGCTTATTTACGGATCGAGGACTGCATCATTGACAACGGCAGGCCA
H988_sensitive         TTGCGGCGGCATGATGCTTATTTACGGATCGAGGACTGCATCATTGACAACGGCAGGCCA
H1008_sensitive        TTGCGGCGGCATGATGCTTATTTACGGATCGAGGACTGCATCATTGACAACGGCAGGCCA
ILRAD700_sensitive     TTGCGGCGGCATGATGCTTATTTACGGATCGAGGACTGCATCATTGACAACGGCAGGCCA
                       ************************************************************

H65_resistant          GCGTCCTCTTGCAGGGATATGCAACAATGTGTCAGTACTGTCGGGTCTTTTTGCAGGTTC
H89_resistant          GCGTTCTCTTGCAGGGATATGCAACAATGTGTCAGTACTATCGGGTCTTTTTGCAGGTTC
H988_sensitive         GCGTTCTCTTGCAGGGATATGCAACAATGTGTCAGTACTATCGGGTCTTTTTGCAGGTTC
H1008_sensitive        GCGTTCTCTTGCAGGGATATGCAACAATGTGTCAGTACTATCGGGTCTTTTTGCAGGTTC
ILRAD700_sensitive     GCGTTCTCTTGCAGGGATATGCAACAATGTGTCAGTACTATCGGGTCTTTTTGCAGGTTC
                       **** ********************************** ********************

H65_resistant          GGCGGCGGCAATAGGACTGAGCAAGTTGCTTTAATGTGAGTGAGTTCAAGGTTTGTGAAG
H89_resistant          GGCGGCGGCAATAGGACTGAGCAAGTTGCTTTAATGTGAGTGAGTTCAAGGTTTGTGAAG
H988_sensitive         GGCGGCGGCAATAGGACTGAGCAAGTTGCTTTAATGTGAGTGAGTTCAAGGTTTGTGATG
H1008_sensitive        GGCGGCGGCAATAGGACTGAGCAAGTTGCTTTAATGTGAGTGAGTTCAAGGTTTGTGAAG
ILRAD700_sensitive     GGCGGCGGCAATAGGACTGAGCAAGTTGCTTTAATGTGAGTGAGTTCAAGGTTTGTGAAG
                       ************************************************************ *

H65_resistant          ACTAAACACTTGTTGTTATTATTTTGCCCATGTGATAAAGTGGGTGTTTTGCGGTTGAAC
H89_resistant          ACTAAACACTTGTTGTTATTATTTTGCCCATGTGATAAAGTGGGTGTTTTGCGGTTGAAC
H988_sensitive         ACTAAACACTTGTTGTTATTATTTTGCCCATGTGATAAAGTGGGTGTTTTGCGGTTGAAC
H1008_sensitive        ACTAAACACTTGTTGTTATTATTTTGCCCATGTGATAAAGTGGGTGTTTTGCGGTTGAAC
ILRAD700_sensitive     ACTAAACACTTGTTGTTATTATTTTGCCCATGTGATAAAGTGGGTGTTTTGCGGTTGAAC
                       ************************************************************

H65_resistant          CACTCATGCTTTTGGTGCACAATTACGTAGCAATGCGTGGCTGAAAGAAGTTATTCATCA
H89_resistant          CACTCATGCTTTTGGTGCACAATTACGTAGCAATGCGTGGCTGAAAGAAGTTATTCATCA
H988_sensitive         CACTCATGCTTTTGGTGCACAATTACGTAGCAATGCGTGGCTGAAAGAAGTTATTCATCA
H1008_sensitive        CACTCATGCTTTTGGTGCACAATTACGTAGCAATGCGTGGCTGAAAGAAGTTATTCATCA
ILRAD700_sensitive     CACTCATGCTTTTGGTGCACAATTACGTAGCAATGCGTGGCTGAAAGAAGTTATTCATCA
                       ************************************************************

H65_resistant          ATTCGTGA
H89_resistant          ATTCGTGA
H988_sensitive         ATTCGTGA
H1008_sensitive        ATTCGTGA
ILRAD700_sensitive     ATTCGTGA
                       ********
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The fact that some polymorphisms were observed in sequencing and not in SSCP could be explained by the higher resolution of the former technique. However, as stated previously, no conserved polymorphism was observed between sensitive and resistant strains.

The results of the sequencing performed on the other nucleoside transporters of *T. vivax* are shown in the Annex 1 of the thesis.

3.4. Conclusion

Despite using highly sensitive methods for the screening of *T. vivax* samples previously characterized for their sensitivity/resistance to DA in goats, our results might suggest that the observed DA-resistance phenotype in two *T. vivax* field samples is not linked to sequence alterations in the nine *T. vivax* genes that are predicted to encode for nucleoside transporters. Our results are based on a limited set of sequencing data and would advantageously be completed by whole genome sequencing on more than 4 strains. Further work is undoubtedly needed to better understand the mechanism of DA-resistance in *T. vivax.* The lack of DNA of high purity did not allow us to use gene walking methods for the gene fragment TvY486_1103760, as this could have permitted to complete the partial sequence to a complete open reading frame for further cloning and analysis.

After improving the performance of an existing molecular tool for DA-resistance diagnosis in *T. congolense* and prospecting without success so far the development of such of tool for *T. vivax*, it would be interesting to explore the current impact of TDR on the health status of trypanosome-infected animals. Indeed, some studies suggest that even in the presence of proven drug resistance, the impact of the parasite on the health of infected cattle could be very limited when the animal is treated with PCV values remaining in a physiological range (Chitanga et al., 2011; Delespaux et al., 2010). This will be the topic of the next chapter of this thesis.

3.5. Reference List

Andrikaye, J.P., 2008. Development of a molecular tool for the detection of resistance against diminazene aceturate in *Trypanosoma vivax*. In. Institute of Tropical Medicine of Antwerp (ITM), pp. 1-36.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J., Delespaux, V., 2011. High Prevalence of Drug Resistance in Animal Trypanosomes without a History of Drug Exposure. PLoS Negl. Trop. Dis. 5 (12): e1454. doi:10.1371/journal.pntd.0001454.

de Koning, H.P., Anderson, L.F., Stewart, M.L., Burchmore, R.J.S., Wallace, L.J., Barrett, M.P., 2004. The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in african trypanosomes. Antimicrob. Agents Chemother. 48, 1515-1519.

Delespaux, V., Chitanga, S., Geysen, D., Goethals, A., Van den Bossche, P., Geerts, S., 2006. SSCP analysis of the P2 purine transporter TcoAT1 gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Trop. 100, 96-102.

Delespaux, V., Vitouley, S.H., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Geerts, S., Van den Bossche, P., 2010. Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracycline and enrofloxacin. PLoS Negl. Trop. Dis. 4, 2-8.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171-180.

Jackson, A.P., Allison, H.C., Barry, J.D., Field, M.C., Hertz-Fowler, C., Berriman, M., 2013. A Cell-surface Phylome for African Trypanosomes. PLoS Negl. Trop. Dis. 7 (3): e2121. doi:10.1371/journal.pntd.0002121.

Kalu, A.U., Oboegbulem, S.I., Uzoukwu, M., 2001. Trypanosomosis in small ruminants maintained by low riverine tsetse population in central Nigeria. Small Rumin. Res. 40, 109-115.

Maser, P., Lüscher, A., Kaminsky, R., 2003. Drug transport and drug resistance in African trypanosomes. Drug Resist. Update 2003, 281-290.

Nakayima, J., Nakao, R., Alhassan, A., Sugimoto, C., 2012. Molecular epidemiological studies on animal trypanosomiases in Ghana. Parasit. Vectors. 5, 217.

Njiokou, F., Simo, G., Nkinin, S.W., Laveissiere, C., Herder, S., 2004. Infection rate of *Trypanosoma brucei* sp., *T. vivax*, *T. congolense* "forest type", and *T. simiae* in small wild vertebrates in south Cameroon. Acta Trop. 92, 139-146.

Rozen, S., Skaletsy, H., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics methods and protocols: methods in molecular biology. Totowa,NJ: Humana Press, pp. 365-86.

Solano, P., Michel, J.F., Lefrancois, T., de La Rocque, S., Sidibe, I., Zoungrana, A., Cuisance, D., 1999. Polymerase chain reaction as a diagnosis tool for detecting trypanosomes in naturally infected cattle in Burkina Faso. Vet. Parasitol. 86, 95-103.

Vitouley, H.S., Mungube, E.O., Allegye-Cudjoe, E., Diall, O., Bocoum, Z., Diarra, B., Randolph, T.F., Bauer, B., Clausen, P.H., Geysen, D., Sidibe, I., Bengaly, Z., Van den Bossche, P., Delespaux, V., 2011. Improved PCR-RFLP for the Detection of Diminazene Resistance in *Trypanosoma congolense* under Field Conditions Using Filter Papers for Sample Storage. PLoS Negl. Trop Dis. 5(7):e1223. doi:10.1371/journal.pntd.0001223.

Vitouley, S.H., Sidibe, I., Bengaly, Z., Marcotty, T., Van den Abbeele, J., Delespaux, V., 2012. Is trypanocidal drug resistance a threat for livestock health and production in endemic areas? Food for thoughts from Sahelian goats infected by *Trypanosoma vivax* in Bobo Dioulasso (Burkina Faso). Vet. Parasitol. 190, 349-354.

Chapter 4

Is trypanocidal drug resistance a threat for livestock health and production in endemic areas? Food for thoughts from Sahelian goats infected by *Trypanosoma vivax* in Bobo Dioulasso (Burkina Faso).

Adapted from

H.S. Vitouley, I. Sidibé, Z. Bengaly, T. Marcotty, J. Van Den Abbeele, V. Delespaux (2012). Is trypanocidal drug resistance a threat for livestock health and production in endemic areas? Food for thoughts from Sahelian goats infected by *Trypanosoma vivax* in Bobo Dioulasso (Burkina Faso). *Veterinary Parasitology* 190, 349-354.

4.1. Introduction

The diagnosis of relapses after treatment is mainly done by microscopic examination, which seriously underestimates the effective relapsing rate, and more seldom by existing molecular tools (Gall et al., 2004). A noticeable difference in sensitivity between the two techniques for the detection of relapses after treatment was already described in a mice model (Chitanga et al., 2011). Furthermore, it was shown that the fluctuating low parasitaemia following the treatment of cattle with ISM after inoculation with ISM-resistant strains of *T. congolense* (Delespaux et al., 2010) had also limited impact on the PCV of the parasitaemic animals. From those observations, the hypothesis was made that latent low parasitaemia infections caused by drug resistant trypanosomes were actually compatible with the survival and an acceptable productivity of their host.

The objectives of this study were thus (i) to evaluate the sensitivity of local *T. vivax* strains to the two main trypanocidal drugs used in the field i.e. ISM and DA, and (ii) to compare the effects of the treatments on the haematocrit and the body weight in animals relapsing after diagnosis by microscopical examination or diagnosis with a trypanosome specific 18S-PCR and in animals completely cleared from parasites.

4.2. Materials and methods

4.2.1. Experimental animals

60 female goats aged between 1 and 3 years, belonging to the Sahelian breed were selected from the North-Eastern region of Burkina Faso (Dori), an area free of tsetse flies (Bengaly et al., 2001; Courtin et al., 2010). The goats were housed in fly-proof facilities. Before the experiment, they were treated with DA at the blanking dose of 7 mg/kg b.w. (i.e. the double of the dose recommended by the manufacturer) and dewormed with oxfendazole (4.5 mg/kg b.w.). Due to endemic pasteurellosis in the area, the animals were vaccinated against this disease and then quarantined for a month. They were fed with fresh or dry straw, supplemented with cotton seed and watered ad libitum with tap water during the quarantine and the experiment. A trypanosome specific 18S-PCR-RFLP (Delespaux et al., 2003; Geysen et al., 2003) was performed on buffy coats sampled from each goat to verify the absence of trypanosomes at the moment of inoculation.

4.2.2. Trypanosome isolates

Twelve isolates were sampled in 6 villages located in the vicinity of Bobo Dioulasso, Burkina Faso (see GIS coordinates in Table 4.1). Species diagnosis was performed using a trypanosome specific 18S-PCR-RFLP (Delespaux et al., 2003; Geysen et al., 2003). Trypanosome-infected blood samples were cryopreserved in a 5% final concentration of DMSO and stored in liquid nitrogen until characterization. Details on those strains are provided in table 4.2.

Table 4.1: GIS coordinates of the sampling sites

Village	X coordinate	Y coordinate
Dafinso	−4.22493	11.28616
Débé	−4.47417	12.03837
Dèrè	−4.28566	11.15875
Kadomba	−4.00108	11.50257
Kangotenga	−3.16267	12.60502

Table 4.2: *T. vivax* isolates used in the *in vivo* goat sensitivity test

Group	Code[a]	Host	Village
1	KAD 41 Tv/F, 10/11/10	Bovin	Kadomba
2	D 39 Tv/F, 13/11/10	Bovin	Dafinso
3	D 42 Tv/F, 13/11/10	Bovin	Dafinso
4	K 56 Tv/F, 12/11/10	Bovin	Koumbia
5	K 30 Tv/F, 8/11/10	Bovin	Koumbia
6	K 4 Tv/F, 8/11/10	Bovin	Koumbia
7	D 32 Tv/F, 13/11/10	Bovin	Dafinso
8	K 28 Tv/F, 8/11/10	Bovin	Koumbia
9	DE 35 Tv/Ch.T3, 02/11/09	Caprin	Dèrè
10	DEB 53 Tv/F, 09/04/09	Bovin	Débé
11	KA 1 Tv/F, 08/04/09	Bovin	Kangotenga
12	DE 57 Tv/F, 03/04/09	Bovin	Dèrè

[a] All strains collected for the Centre International de Recherche-Développement sur l'Elevage en zone Subhumide (CIRDES)

4.2.3. Experimental infection

Each of the twelve *T. vivax* cryostabilates was thawed at 37°C and reactivated through a passage in a goat. Parasitaemia was monitored by microscopical examination of the buffy coat as described by Murray et al. (1977). At the first peak of the parasitaemia, blood was collected and diluted with PBS/Glucose (5%) to a final concentration of 10^5 trypanosomes / ml.

For each isolate, five goats were ear-tagged and inoculated intravenously with 10^5 reactivated trypanosomes. At the first observation of parasites in the blood of one of the five animals of a group, two were treated with 3.5 mg/kg b.w. DA, two with 0.5 mg/kg b.w. ISM and one left untreated as control. A dose of 7 mg/kg b.w. DA, i.e. the double of the dose recommended by the manufacturer to clear *T. vivax* infection in domestic animals was used for goats showing a life-threatening PCV (below 15%) during the observation period. For these animals, observations were stopped at that moment.

4.2.4. Monitoring

After inoculation, the goats were parasitologically monitored every 5 days by sampling blood from the jugular vein with heparinized Vacutainer® tubes (BD Medical). From those tubes, blood was collected into glass capillary tubes without anti-clotting agent and stopped at an extremity with Cristaseal® (Hawxley). After centrifugation at 9000 rpm for 5 min, the values of the hematocrit were recorded. Buffy coats were examined using a microscope (x400) for the detection of trypanosomes. A slide was considered as negative if no trypanosome was observed in 50 fields. For each animal, one buffy coat was placed on a filter paper (Whatman N°4, Whatman®), dried protected from UV light, stored in individual envelopes that were placed in plastic bags containing silica gels and conserved at -20°C for subsequent molecular analysis with the trypanosome specific 18S-PCR-RFLP (Delespaux et al., 2003; Geysen et al., 2003). The RFLP step necessary for the trypanosome species determination was conserved, even if the species that was inoculated was known, to confirm the specificity of the PCR reaction. The DNA stored on the filter papers was extracted using the routine PBS-Saponin technique (de Almeida et al., 1997). The parasitaemia, the haematocrit and the body weight of the goats were controlled every 5 days after treatment with the trypanocides for a period of 100 days.

4.2.5. Statistical analysis

PCV data were analyzed using cross-sectional time-series linear regressions in Stata 10, separately for DA and ISM treatments. Categorical explanatory variables were the goat status (no relapse, relapse at microscopy or relapse at PCR), the period of observation (<15, 15-44 or ≥45 days post-infection) and the interactions between the two. Individual goats were considered as random effects. Finally, relative PCV differences between periods (using period 1 as denominator) were calculated using the non-linear combination of estimators (delta method). The difference of relative differences was calculated using the following equations:

$$DRD_{PCV} = \left(\frac{PCV_{Rx} - PCV_{R1}}{PCV_{R1}}\right) - \left(\frac{PCV_{NRx} - PCV_{NR1}}{PCV_{NR1}}\right)$$

$$DRD_{W} = \left(\frac{W_{RX} - W_{R1}}{W_{R1}}\right) - \left(\frac{W_{NRX} - W_{NR1}}{W_{NR1}}\right)$$

Where DRD is the difference of relative differences; PCV the packed cell volume; W the weight; NR the non-relapsing goats; R the relapsing goats; 1 the Period 1 or day 1 and x is the period 2 or 3 or day 2, 3, or 4. Furthermore, weight data were analyzed in similar models, except that explanatory variables were the goat's status, time of observation (days 0, 30, 60 and 90 post-infection) and the interaction between the two.

4.3. Results

4.3.1. Health condition

Control and treated goats presenting severe clinical signs or a PCV below 15 were treated with DA 7 mg/kg b.w. which caused in all cases an increase of the PCV back to physiological values or at least values higher than 20%. Nine goats from 6 ISM groups (1, 2, 5, 9, 10 and 11) and the 2 goats from DA group 2 had to be treated with a high dose of DA to rescue them from certain death. This corresponds to all relapses diagnosed by microscopic examination (see Table 4.3).

4.3.2. Sensitivity tests of the *T. vivax* strains

Among the 12 groups of four goats (control not included) that were screened by microscopical examination, six were completely cured after treatment, 5 showed relapses in at least one goat treated with ISM and 1 showed relapses in all treated goats. The data are summarized in table 4.3.

Table 4.3: Results of the microscopic examination of the 60 goats

	DA1	DA2	ISM1	ISM2	C
Group 1	-		+	-	+
Group 2	+	+	+	+	+
Group 3	-	-	-	-	+
Group 4	-	-	-	-	+
Group 5	-	-	+	-	+
Group 6	-	-	-	-	+
Group 7	-	-	-	-	+
Group 8	-	-	-	-	+
Group 9	-	-	-	+	+
Group 10	-	-	+	+	+
Group 11	-	-	+	+	+
Group 12	-	-	-	-	+

With + as animal found microscopically positive during the examination period, DAx goat treated with DA, ISMx goat treated with ISM and C as control.

For the 6 groups that were randomly selected and additionally screened with the trypanosome specific 18S-PCR-RFLP, the following results were observed: for the groups treated with DA no relapses by microscopic examination, and 83.3% (10/12) using the 18S-PCR-RFLP. For the groups treated with ISM, 25% (3/12) relapses by microscopic examination and 83.3% (10/12) with the 18S-PCR. The data are summarized in table 4.4.

Table 4.4: Comparison of the results of the microscopic examination and the 18S-PCR for goats

	Microscope				PCR			
	DA1	DA2	ISM1	ISM2	DA1	DA2	ISM1	ISM2
Group 1	N	N	6x	N	3x	3x	6x	4x
Group 3	N	N	N	N	4x	N	3x	N
Group 4	N	N	N	N	2x	3x	1x	N
Group 5	N	N	N	8x	3x	3x	3x	8x
Group 6	N	N	N	N	3x	5x	3x	3x
Group 9	N	N	N	8x	2x	N	1x	8x
Total relapses	0/6	0/6	1/6	2/6	6/6	4/6	6/6	4/6

With N as negative, XX as number of times that an animal was observed positive during the 100 days observation period (every 5 days), DAx goat treated with DA and ISMx goat treated with ISM.

4.3.3. PCV and weight evolution of DA relapsing goats compared to DA non-relapsing goats

The relative average PCV in goats that relapsed microscopically, decreased significantly more than in non-relapsing goats. The difference between the relative average PCV reduction between period 1 (day 0–14) and period 2 (day 15–44) in relapsing and non-relapsing goats was estimated at 24.9% (95% CI 17.9-31.9%). This difference was not significant when relapses were detected using the trypanosome specific 18S-PCR-RFLP and this when comparing period 1 to period 2 or period 1 to period 3 (≥45 days). No difference was observed in the weight evolution between relapsing or non-relapsing goats and this for all comparisons in time i.e. time 1 versus 2, 1 versus 3 and 1 versus 4. The results for the weight evolution are summarized in table 4.5.

4.3.4. PCV and weight evolution of ISM relapsing goats compared to ISM non-relapsing goats

The relative average PCV in goats that relapsed microscopically, decreased significantly more than in non-relapsing goats. The difference between the relative average PCV reduction period 1 and period 2 in relapsing and non-relapsing goats was estimated at 10.1% (95% CI 4.8-15.4%). This difference was also significant when relapses were detected using the trypanosome specific 18S-PCR-RFLP (difference of average PCV

reduction between period 1 and period 2 in relapsing and non-relapsing goats: 8.4-95% CI 14.4-2.4% and difference of average PCV reduction between period 1 and period 3: 12.5-95% CI 7.2-17.7%). The goats that relapsed microscopically presented significantly higher relative weight losses for all the three recording times compared to day 0. Relapses goats at 18S-PCR-RFLP did not present significantly higher relative weight loss than non-relapsing goats and this for the three recording times. The results are summarized in table 4.6 below.

Table 4.5: Weight evolutions of relapsing goats compared to non-relapsing after treatment with DA with probabilities and 95% confidence intervals

Comparison	Observed reduction	Probability	95% low	95% up
Micro 1-2	3.73%	0.44	-5.70%	13.16%
Micro 1-3	-6.05%	0.21	-15.51%	3.40%
Micro 1-4	-7.58%	0.12	-17.08%	1.91%
PCR 1-2	2.27%	0.46	-3.80%	8.33%
PCR 1-3	1.97%	0.53	-4.11%	8.05%
PCR 1-4	0.75%	0.81	-5.33%	6.83%

Difference of the relative weight reduction (using weight at time 1 as denominator) in relapsing and non-relapsing goats. With Micro x-x as the comparison of the relative weight loss of animals relapsing microscopically between period x and x; PCR x-x as comparison of the relative weight loss of animals relapsing when examined by PCR between period x and x.

Table 4.6: Weight evolutions of relapsing goats compared to non-relapsing after treatment with ISM with probabilities and 95% confidence intervals

Comparison	Observed reduction	Probability	95% low	95% up
Micro 1-2	-9.38%	0.01	-16.35%	-2.40%
Micro 1-3	-14.96%	0.00	-22.00%	-7.92%
Micro 1-4	-17.14%	0.00	-24.22%	-10.06%
PCR 1-2	-2.43%	0.53	-10.03%	5.17%
PCR 1-3	-2.98%	0.45	-10.67%	4.70%
PCR 1-4	-2.60%	0.51	-10.37%	5.17%

Difference of the relative weight reduction (using weight at time 1 as denominator) in relapsing and non-relapsing goats. With Micro x-x as the comparison of the relative weight loss of animals relapsing microscopically between period x and x; PCR x-x as comparison of the relative weight loss of animals relapsing when examined by PCR between period x and x.

4.4. Discussion

Animal health and production in zones that are endemic for trypanosomosis have always be challenging from an economic perspective. Decreasing the impact of the disease on the health status of their animals is often the only possible strategy for farmers with scarce financial resources. Some farmers act strategically by preferring trypanotolerant animals in net-protected units (Bauer et al., 2006). Yet, in most cases, farmers rely on trypanocidal drugs that are administered with different levels of know-how (Grace et al., 2009) but also often without any parasitological diagnosis or even clinical examination (Van den Bossche et al., 2000). The sole trypanocides available for animal use have been marketed for more than half a century. Considering the nearly blind and routine administration of those products, it is not astonishing that drug resistance prevails in many regions of Africa (Delespaux et al., 2008). Even if constructive initiatives were implemented for promoting a rational use of trypanocides (Liebenehm et al., 2011), the prevailing situation is described by many authors as very alarming (Chaka and Abebe, 2003; Mamoudou et al., 2008). However, an experimental study in a mice model where ISM-resistant trypanosomes were inoculated into ISM-treated and untreated animals suggests that even when TDR is evident, it might be relevant to threat the infected animals (Delespaux et al., 2010).

From this study on the sensitivity of different *T. vivax* isolates to the two main trypanocidal drugs used in the field i.e. ISM and DA, we can conclude that drug resistance for these parasites is present but still manageable in the region around Bobo Dioulasso as only 1/12 isolate was found resistant to both drugs and 5/12 resistant to ISM only allowing in most instances the use of the sanative pair. The multi-resistant isolate albeit highly virulent was cleared microscopically with 7mg/kg b.w. of DA, i.e. the double of the dose recommended by the manufacturer to clear *T. vivax* infection in domestic animals. The presence of drug resistance in *T. vivax* in Burkina Faso being confirmed, the second aim of this study was to evaluate the effects of the treatment in animals inoculated and treated at the first observation of parasites and to compare the evolution of the weight and PCV in (i) microscopically relapsing animals, (ii) animals relapsing when diagnosed by the trypanosome specific 18S-PCR-RFLP and finally (iii)

animals completely cured and this in controlled conditions but the closest possible to field conditions.

As reasonably expected, the relative PCV reductions were significantly higher in animals relapsing microscopically i.e. in animals showing the highest parasitaemia. For the relapsing animals diagnosed using the trypanosome specific 18S-PCR-RFLP i.e. animals with the lowest parasitaemia, the fact that the relative PCV reductions were only significant after treatment with ISM, suggests a higher degree of ISM-resistance compared to DA. A lower level of resistance against DA would allow the host immunity together with the temporary toxic effect of the drug to keep the parasite under control. This might not be the case with ISM for which higher degree of resistance would not allow animals to control the parasite as efficiently as with DA in spite of the fact that the elimination of ISM from the host is far slower (approximately 2 months compared to two weeks for DA). Yet, the absence of significant effect in DA treated animals could be attributed to the low number of relapses with DA. Interestingly, the animals showing a relapse with a high parasitaemia (microscopic diagnosis) presented the highest relative PCV reductions (24.9% and 10.1% after treatment with DA and ISM respectively). It can be reasonably assumed that, as it was observed in *T. congolense* (Masumu et al., 2006), the virulence of different strains of *T. vivax* might vary and, among the drug resistant parasites, the most virulent only will cause visible deleterious effects on the health of the relapsing animals. Furthermore, self-cure appears to be relatively common in *T. vivax* infections (Gardiner, 1989) and it was observed that animals that recovered spontaneously from an acute infection developed chronic and asymptomatic infections (Batista et al., 2009). The treatment with trypanocide combined with the immune system of the host could increase this recovery rate by allowing a better control of the parasite and resulting in very low oscillating parasitaemia with no or little impact on the host's health. Furthermore, as hypothesized above for PCV, the significantly higher weight losses in ISM relapsing animals (diagnosed using microscopy i.e. with the highest parasitaemia) might be explained by a higher resistance against this particular drug compared to DA.

4.5. Conclusion

This study showed that TDR might probably have limited impact on the PCV and body weight losses of experimental goats infected with *T. vivax*, except for a few highly virulent strains. However, this still needs to be confirmed in cattle under natural tsetse and *Trypanosoma* challenge. Nevertheless, it would be crucial to find an alternative in order to contain or reverse the TDR phenomenon in West Africa.

4.6. Reference List

Batista, J.S., Oliveira, A.F., Rodrigues, C.M.F., Damasceno, C.A.R., Oliveira, I.R.S., Alves, H.M., Paiva, E.S., Brito, P.D., Medeiros, J.M.F., Rodrigues, A.C., Teixeira, M.M.G., 2009. Infection by *Trypanosoma vivax* in goats and sheep in the Brazilian semiarid region: From acute disease outbreak to chronic cryptic infection. Vet. Parasitol. 165, 131-135.

Bauer, B., Gitau, D., Oloo, F.P., Karanja, S.M., 2006. Evaluation of a preliminary title to protect zero-grazed dairy cattle with insecticide-treated mosquito netting in western Kenya. Trop. Anim. Health Prod. 38, 29-34.

Bengaly, Z., Ganaba, R., Sidibe, I., Desquesnes, M., 2001. Trypanosomose animale chez les bovins dans la zone Sud-soudanienne du Burkina Faso. Résultats d'une enquête sérologique. Revue Elev. Méd. Vét. Pays trop. 54, 221-224.

Chaka, H., Abebe, G., 2003. Drug resistant trypanosomes : a threat to cattle production in the southwest of Ethiopia . Revue Elev. Méd. Vét. Pays trop. 56, 33-36.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van den Abbeele, J., Delespaux, V., 2011. High Prevalence of Drug Resistance in Animal Trypanosomes without a History of Drug Exposure. PLoS Negl. Trop. Dis. 5.

Courtin, F., Rayaisse, J.B., Tamboura, I., Serdebeogo, O., Koudougou, Z., Solano, P., Sidibe, I., 2010. Updating the northern tsetse limit in Burkina Faso (1949-2009): impact of global change. Int. J. Environ. Res. Public Health 7, 1708-1719.

de Almeida, P.J., Ndao, M., Van, M.N., Geerts, S., 1997. Diagnostic evaluation of PCR in goats experimentally infected with *Trypanosoma vivax*. Acta Trop. 66, 45-50.

Delespaux, V., Ayral, F., Geysen, D., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification: applicability for the diagnosis of mixed infections with different trypanosome species in cattle. Vet. Parasitol. 117, 185-193.

Delespaux, V., Geysen, D., Van den Bossche, P., Geerts, S., 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol. 24, 236-242.

Delespaux, V., Vitouley, S.H., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Geerts, S., Van den Bossche, P., 2010. Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracycline and enrofloxacin. PLoS Neglect. Trop. Dis. 4, 2-8.

Gall, Y., Woitag, T., Bauer, B., Sidibe, I., McDermott, J., Mehlitz, D., Clausen, P.H., 2004. Trypanocidal failure suggested by PCR results in cattle field samples. Acta Trop. 92, 7-16.

Gardiner, P.R., 1989. Recent studies of the biology of *Trypanosoma vivax*. Adv. Parasitol 28, 229-317.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171-180.

Grace, D., Randolph, T., Affognon, H., Dramane, D., Diall, O., Clausen, P.H., 2009. Characterisation and validation of farmers' knowledge and practice of cattle trypanosomosis management in the cotton zone of West Africa. Acta Trop. 111, 137-143.

Liebenehm, S., Affognon, H., Waibel, H., 2011. Collective livestock research for sustainable disease management in Mali and Burkina Faso. Int. J. Agr. Sustain. 9, 212-221.

Mamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S., 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. Acta Trop. 106, 115-118.

Masumu, J., Marcotty, T., Geysen, D., Geerts, S., Vercruysse, J., Dorny, P., Van den Bossche, P., 2006. Comparison of the virulence of *Trypanosoma congolense* strains isolated from cattle in a trypanosomiasis endemic area of eastern Zambia. Int. J. Parasitol. 36, 497-501.

Murray, M., Murray, P.K., Mcintyre, W.I.M., 1977. Improved parasitological technique for diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 71, 325-326.

Van den Bossche, P., Doran, M., Connor, R.J., 2000. An analysis of trypanocidal drug use in the Eastern Province of Zambia. Acta Trop. 75, 247-258.

Chapter 5

Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracyclines and enrofloxacin.

Adapted from:

Delespaux V., Vitouley H.S*., Marcotty T., Speybroeck N., Berkvens D., Roy K., Geerts S., Van den Bossche P. (2010). Chemosensitization of *Trypanosoma congolense* Strains Resistant to Isometamidium Chloride by Tetracyclines and Enrofloxacin. PLoS Negl Trop Dis 4(9): e828. doi:10.1371/journal.pntd.0000828

* Author contribution:
- Performed the experiment in cattle and all PCR's
- Compiled and analyzed the cattle's and PCR's data
- Participated in the redaction of the manuscript by writing the paragraph on the experiment in cattle and PCR analysis
- Participated in the reading and the correction of the entire manuscript

5.1. Introduction

Considering the wide distribution of TDR previously mentioned, strategic alternatives are urgently needed to circumvent this plague. Reversal of drug resistance or chemosensitization was successfully achieved, among others, in yeast (Knorre et al., 2009), *Plasmodium* (Bhattacharjee et al., 2001; Masseno et al., 2009), cancer cells (Tao et al., 2009) and *Leishmania* (Wong et al., 2009). Such strategies could bring a much needed relief to African livestock breeders if they could be implemented at a reasonable price by shortcutting the development of new compounds, toxicity studies and long clinical trials. Many bacterial secondary multidrug resistance transporters belonging to the two major families, i.e. the Major Facilitator Superfamily (MFS) and the Multi Antimicrobial Extrusion Family (MatE) are described as having affinity for ethidium bromide (Homidium) as well as for many different compounds such as plant alkaloids, noxious metabolic products (such as fatty acids or bile salts), organic solvents and diverse antibiotics (Mazurkiewicz et al., 2005). At least eight representatives of those transporter families are present in the genome of *T. congolense*. Homidium is part of the ISM molecule, the structural relatedness of both molecules being thus obvious (Figure 5.1). Furthermore, in the field, cross-resistance is observed between ethidium bromide and ISM (Olila et al., 2002) suggesting that uptake and extrusion of the drug within and from the trypanosome are mediated by the same mechanisms for both compounds.

In a preliminary experiment, a number of antibiotics were selected and screened in a mouse model consisting of the inoculation of ISM-resistant trypanosomes into ISM-treated and untreated animals (Table 5.1) (Delespaux et al., 2010). The working hypothesis was that chemical compounds could interfere (compete) with the extrusion of ISM from the drug resistant trypanosome allowing a prolonged trypanocidal action. The criterion for inclusion in this study was the affinity of the medications for bacterial efflux systems as described for ß-lactams (Li et al., 1994; Pages et al., 2009), tetracycline (TC), oxytetracycline (OTC) (Roberts, 2005), nalidixic acid (quinolone) (Bailey et al., 2008) and the fluoroquinolone enrofloxacine (FQE) (Usui et al., 2009). After this preliminary screening, TC was chosen as the easiest and cheapest commercial preparation for oral administration to mice by dilution in drinking water. Despite the unusual high degree of resistance of the *T. congolense* strain used in this study, the

survival times were significantly higher after treatment with the association of ISM and TC. When considering the efficacy of the compounds against the trypanosomes, the complete ineffectiveness of TC alone and the increased efficacy of ISM in the presence of TC, provides strong arguments in favor of the hypothesis that the two compounds compete for the same efflux system.

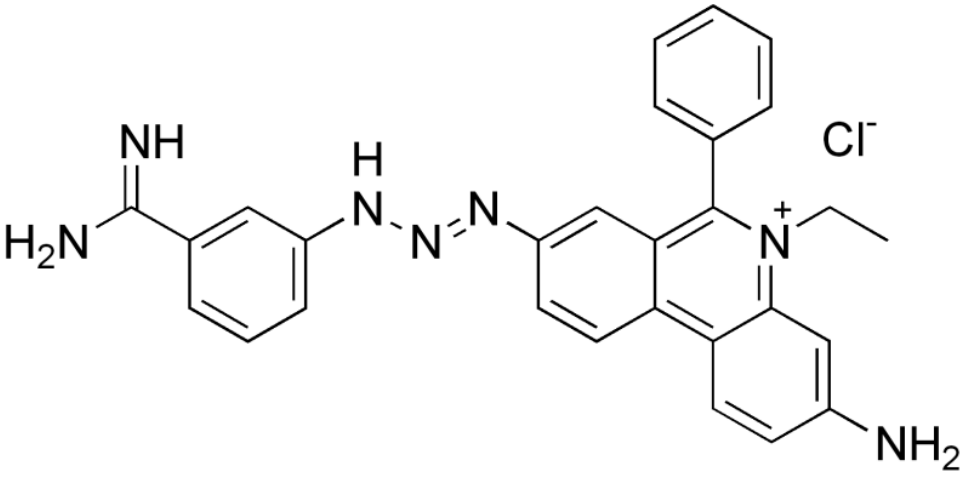
The objective of the work presented in this chapter was to study the effect of some antibiotics on the efficacy of ISM in cattle infected with ISM-resistant *T. congolense* resistant infected animals. From the preliminary experiment in mice, OTC was selected for the experiment in cattle as it is available as an injectable long acting form allowing for a reduction of the number of injections. Enrofloxacin was not pre-tested in combination with ISM in mice but immediately used in cattle.

Table 5.1: Initial screening of antibiotics in a mouse model (*T. congolense* strain IL3343)

Treatment	Cured	Median PP (days)	95% confidence intervals
Untreated control	0/6	5,41	4,10-7,16
Penicilline G (100mg/kg)	0/6	5,79	4,38-7,77
Nalidixic acid (175mg/kg)	0/6	6,29	4,76-8,32
Tetracycline (125mg/kg)	0/6	6,80	5,14-8,99
Oxytetracycline (125mg/kg)	0/6	5,06	3,83-6,69
Enrofloxacine (40mg/kg)	0/6	5,22	3,95-6,90
ISM (1mg/kg)	0/6	16,29	12,32-21,53
ISM/Penicilline G (*)	0/6	12,27	9,28-16,22
ISM/Nalidixic acid	0/6	16,73	12,66-22,13
ISM/Tetracycline	4/6	51,96	38,01-71,05
ISM/Oxytetracycline	3/6	39,1	29,16-52,43
ISM/Enrofloxacine	N.T.	-	-

(*) ISM at 1mg/kg combined to the antibiotic at the dose under used alone; N.T.: Not Tested

Isometamidium



Homidium

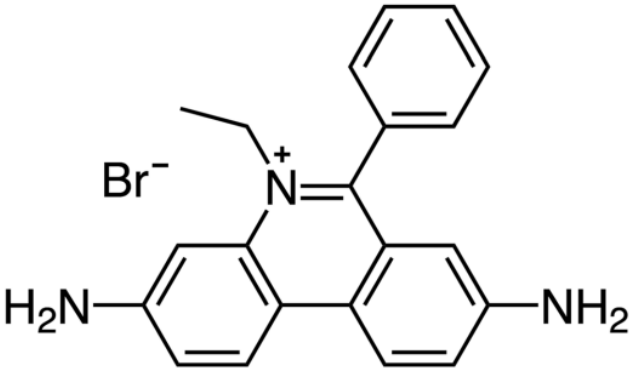


Figure 5.1: Structural relatedness between ISM and ethidium salts

5.2. Materials and methods

5.2.1. Trypanosome strains

The cloned *T. congolense* savannah type strain IL3343 used to infect cattle was identified as resistant to ISM when tested in mice (CD50 = 1.7 mg/kg) (Peregrine et al., 1997). The CD50 is defined as the curative dose that gives complete cure in 50% of the animals.

5.2.2. Cattle inoculation and treatment

Three groups of 6 adult crossbred zebus weighing on average 158 kg each (extremes 140 and 201kg) were inoculated with 5 x 10^5 trypanosomes (cloned isolate IL3343) each by intra-jugular injection 30 days after treatment with DA (7mg/kg) to clear all trypanosomal infections and deworming. One non-treated control group of 2 cattle was inoculated in the same way. The 20 cattle were housed in fly-proof facilities. From day 7 after the inoculation, all animals were monitored 2 times a week during 95 days. Their PCV was measured and jugular blood was examined for the presence of parasites by microscopic examination of the buffy coats and by PCR (Geysen et al., 2003) performed on buffy coats collected on Whatman 4 filter paper (Whatman). The DNA was obtained using a routine chelex-based extraction method (Sambrook et al., 1989).

At the first parasitaemia, group A was treated with one single administration of 0.5mg/kg ISM by intramuscular (IM) injection, group B with one single administration of 0.5mg/kg ISM and with 20mg/kg OTC (Terramycin LA) IM every 3 days for 30 days and group C with one single administration of 0.5mg/kg ISM and with 5mg/kg FQE (Baytril 100) IM every 2 days for 30 days. For each animal, the injection sites of the drugs were alternatively selected in forehand and hindquarters, shaved and colored with methylene blue and picric acid for OTC and FQE respectively. A minimal distance of 6 cm between injection sites was respected.

5.2.3. Statistical analysis

The cattle's PCV values were analyzed using a cross-sectional linear regression, accounting for repeated measures from individual animals in Stata 10 (Copyright 1996–2009 StataCorp LP). Explanatory variables were the animal groups, post-treatment

periods and the interactions between them. Three post-treatment periods, each containing the same number of samplings, were defined as follows: day 1–21, day 22–54 and day 55–95. The interaction term between the groups and the third period (using the first period as a baseline) was used as indicator of the impact of the disease on the PCV.

5.3. Results and discussion

The two untreated control animals became parasitaemic 11 days after inoculation and were treated with DA (7 mg/kg) on day 30 because their PCV reached the critical value of 25. All 6 animals of group A (ISM) became positive between days 24 and 46 post-inoculation. The data are summarized in table 5.2. When ISM was used in combination with either OTC (group B) or FQE (group C), the relapse period was significantly longer ($p<0.001$; Figure 5.2). Fifty percent of the cattle became infected (between days 46 and 82) and 50% completely cleared the infection. In the groups B (ISM-OTC) and C (ISM-FQE), the parasitaemia remained very low, below the detection level of the microscopic examination, i.e. 450 trypanosomes / ml (Ancelle et al., 1997). The PCR results were fluctuating with animals being detected parasitaemic every 2 to 3 weeks, indicating a parasitaemia oscillating just above and below the detection limit of the PCR test, i.e. 25 trypanosomes/ml blood (Geysen et al., 2003).

The impact of the infection on the PCV was not very pronounced in the ISM-treated groups, even in group A (average PCV reduction 8 to 14 weeks after treatment: 5.9%; 95% CI: 4.5–7.3). However, this impact was lower in groups B (ISM-OTC) and C (ISM-FQE) compared to group A (ISM) ($p<0.01$). These observations indicate that even in the case of ISM-resistant trypanosomes, farmers still seem to benefit from the use of the trypanocide because of the significant decrease of the effect of the infection on the health status of the animals as represented in the PCV values.

Table 5.2: Summarized data of the output of the treatments in cattle

	Group A (ISM)	**Group B (ISM-OTC)**	**Group C (ISM-FQE)**
Number of animals	6	6	6
Median prepatent period (days)	35 (26-47)	84 (61-117)	91 (66-127)
Mean PCV drop from period 1 to period 3	5.9 (4.5-7.3)	1.8 (0.3-3.2)	3.3 (1.9-4.7)
Cured	0	3	3

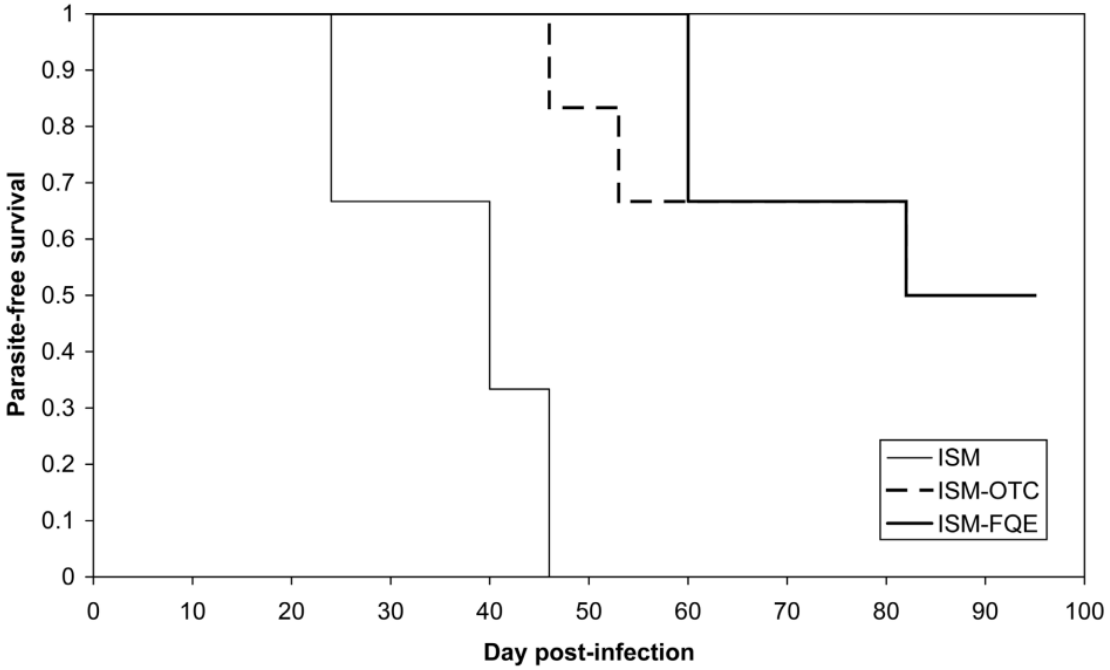


Figure 5.2: Kaplan-Meier survival estimates in cattle infected with the resistant strain IL3343. With ISM: group A; ISM-OTC: group B; ISM-FQE: group C.

5.4. Prospects and impact

Although resistance to DA and ISM is developing quickly (Delespaux and de Koning, 2007; Delespaux et al., 2008; Geerts et al., 2001), controlling the parasite in livestock using drugs remains the control method of choice for small-scale livestock breeders. Localized tsetse control is usually not effective (Vale, 2009) and a vaccine is not yet available, leaving little choice to control the disease. Animal trypanosomosis not only affects livestock production (milk, meat) but also impacts greatly on crop production through the inability to keep draft animals in tsetse-infested areas (Shaw, 2004). Notwithstanding the alarming levels of TDR that have been reported in the cotton belt of West Africa (McDermott et al., 2003) and in some regions of southern Africa (including Zambia) (Delespaux et al., 2008; Mamoudou et al., 2008), new trypanocidal drugs for animal use are not expected to become available in the near future. Hence, potentiating the available trypanocidal drugs may represent a powerful alternative to the current problems associated with the control of trypanosomes in livestock. Research in the field of non-competitive inhibitors of efflux pumps in bacteria is being conducted (Markham, 1999; Sangwan et al., 2008) and may ultimately represent an immense hope for future control of trypanosomosis using drugs. In the meantime, TC and some derivatives are cheap drugs, registered for use in livestock, widely available on the African market and with an expired patent, now in the public domain. More importantly, TC is commonly used by African farmers and will not require elaborate new chemistry and safety tests. Hence, assuming that further trials confirm the effectiveness of the antibiotics in potentiating the activity of trypanocidal drugs in cattle under natural tsetse challenge, the new control approach can be implemented rapidly. It is likely that the combination ISM–TC/OTC can also be made more cost effective after adjusting dosage and the duration of the treatment. Furthermore, several analogues of TC/OTC and FQE are available albeit somewhat more expensive as patents are still in force. These compounds are currently being screened with the aim of optimizing the delivery system to increase the specificity of the treatment, to boost the intracellular concentration of the chemosensitizer within the trypanosome and to reduce the dose. Obviously, the current treatment schedule cannot be used under field conditions. The repeated administration of a high dose of antibiotics is far too expensive for the rural communities and would certainly render the treated animals unsuitable for human consumption. Further

research is thus ongoing to identify the best galenic solution, the optimal combination of chemosensitizer with ISM (qualitative and quantitative) and to test this combination in livestock under controlled and field conditions in areas with high tsetse challenge and high TDR. An effective combination of ISM and chemosensitizer(s) should result in (i) a decrease in the proportion of circulating strains resistant to ISM and (ii) a decrease in the impact of the disease on the health status of the cattle. Strategic use of this approach may result in an increased efficacy of currently available trypanocidal drugs in extensive areas of sub-Saharan Africa where their use is severely curtailed as a result of the development of TDR.

5.5. Reference List

Ancelle, T., Paugam, A., Bourlioux, F., Merad, A., Vigier, J.P., 1997. Detection of trypanosomes in blood by the Quantitative Buffy Coat (QBC) technique: experimental evaluation. Med. Trop. (Mars.) 57, 245-248.

Bailey, A.M., Paulsen, I.T., Piddock, L.J.V., 2008. RamA Confers Multidrug Resistance in *Salmonella enterica* via Increased Expression of *acrB*, Which Is Inhibited by Chlorpromazine . Antimicrob. Agents Chemother. 52, 3604-3611.

Bhattacharjee, A.K., Kyle, D.E., Vennerstrom, J.L., 2001. Structural analysis of chloroquine resistance reversal by imipramine analogs. Antimicrob. Agents Chemother. 45, 2655-2657.

Delespaux, V., de Koning, H.P., 2007. Drugs and drug resistance in African trypanosomiasis. Drug Resist. Update 10, 30-50.

Delespaux, V., Dinka, H., Masumu, J., Van den Bossche, P., Geerts, S., 2008. Five fold increase in the proportion of diminazene aceturate resistant *Trypanosoma congolense* isolates over a seven years period in Eastern Zambia. Drug Resist. Update 11, 205-209.

Delespaux, V., Vitouley, S.H., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Geerts, S., Van den Bossche, P., 2010. Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracycline and enrofloxacin. PLoS Negl. Trop. Dis. 4, 2-8.

Geerts, S., Holmes, P.H., Diall, O., Eisler, M.C., 2001. African bovine trypanosomiasis: the problem of drug resistance. Trends Parasitol. 17, 25-28.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet. Parasitol. 110, 171-180.

Knorre, D.A., Krivonosova, T.N., Markova, O.V., Severin, F.F., 2009. Amiodarone inhibits multiple drug resistance in yeast *Saccharomyces cerevisiae*. Arch. Microbiol. 191, 675-679.

Li, X.Z., Ma, D., Livermore, D.M., Nikaido, H., 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to beta-lactam resistance. Antimicrob. Agents Chemother. 38, 1742-1752.

Mamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S., 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. Acta Trop. 106, 115-118.

Markham, P.N., 1999. Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. Antimicrob. Agents Chemother. 43, 988-989.

Masseno, V., Muriithi, S., Nzila, A., 2009. In vitro chemosensitization of *Plasmodium falciparum* to antimalarials by Verapamil and Probenecid. Antimicrob. Agents Chemother. 53, 3131-3134.

Mazurkiewicz, P., Driessen, A.J.M., Konings, W.N., 2005. What do proton motive force driven multidrug resistance transporters have in common? Curr. Issues Mol. Biol. 7, 7-21.

McDermott, J., Woitag, T., Sidibe, I., Bauer, B., Diarra, B., Ouedraogo, D., Kamuanga, M., Peregrine, A.S., Eisler, M.C., Zessin, K.H., Mehlitz, D., Clausen, P.H., 2003. Field studies of drug-resistant cattle trypanosomes in Kenedougou Province, Burkina Faso. Acta Trop. 86, 93-103.

Olila, D., McDermott, J.J., Eisler, M.C., Mitema, E.S., Patzelt, R.J., Clausen, P.H., Poetzsch, C.J., Zessin, K.H., Mehlitz, D., Peregrine, A.S., 2002. Drug sensitivity of trypanosome populations from cattle in a peri-urban dairy production system in Uganda. Acta Trop. 84, 19-30.

Pages, J.M., Lavigne, J.P., Leflont-Guibout, V., Marcon, E., Bert, F., Noussair, L., Nicolas-Chanoine, M.H., 2009. Efflux pump, the masked side of beta-lactam resistance in *Klebsiella pneumoniae* clinical isolates. PLoS ONE 4, 1-9.

Roberts, M.C., 2005. Update on acquired tetracycline resistance genes. FEMS Microbiol. Lett. 245, 195-203.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Arbor, New York.

Sangwan, P.L., Koul, J.L., Koul, S., Reddy, M.V., Thota, N., Khan, I.A., Kumar, A., Kalia, N.P., Qazi, G.N., 2008. Piperine analogs as potent *Staphylococcus aureus* NorA efflux pump inhibitors. Bioorgan Med. Chem. 16, 9847-9857.

Shaw, P.M.A., 2004. Economics of African trypanosomiasis. In: Maudlin, I., Holmes, P.H., Miles, M.A. (Eds.), The Trypanosomiasis. CABI Publishing, Wallingford, UK, pp. 369-402.Sones, K., 2001. Pharmaceutical companies: partners or enemies ? ICPTV Newsletter 3, 19-21.

Tao, L.Y., Liang, Y.J., Wang, F., Chen, L.M., Yan, Y.Y., Dai, C.L., Fu, L.W., 2009. Cediranib (recentin, AZD2171) reverses ABCB1-and ABCC1-mediated multidrug resistance by inhibition of their transport function. Cancer Chemother. Pharmacol. 64, 961-969.

Usui, M., Uchiyama, M., Iwanaka, M., Nagai, H., Yamamoto, Y., Asai, T., 2009. Intracellular concentrations of enrofloxacin in quinolone-resistant *Salmonella enterica* subspecies *enterica* serovar *Choleraesuis*. Int. J. Antimicrob. Agents 34, 592-595.

Vale, G.A., 2009. Prospects for controlling trypanosomosis. Onderstepoort J. Vet. Res. 76, 41-51.

Wong, I.L.K., Chan, K.F., Zhao, Y.Z., Chan, T.H., Chow, L.M.C., 2009. Quinacrine and a novel apigenin dimer can synergistically increase the pentamidine susceptibility of the protozoan parasite *Leishmania*. J. Antimicrob. Chemother. 63, 1179-1190.

Chapter 6

General Discussion:

The problem of trypanocidal drug resistance in West Africa

6.1. Introduction

The development, the spread and the control of TDR have been the subject of various researches in West Africa (Authie, 1984; McDermott et al., 2003; Grace, 2005; Talaki, 2008; Clausen et al., 2010). In this chapter, we will discuss about the management of TDR in West Africa in a broader context of animal trypanosomosis by taking into account (i) the current distribution and impact of TDR on livestock health and productivity and (ii) the role of molecular tools in the proactive management of TDR.

6.2. Distribution and impact of TDR in West Africa

The establishment of an updated low-resolution data set on the distribution of TDR in West Africa was made possible by the creation of the RESCAO, the monitoring epidemiological network including eight West African countries. Even if the quality of the action was unequal between countries, the harmonization of the protocols and further training of the administrative and technical stakeholders will allow for more comparable results in the future. The structure and the technical staff being in place, all procedures have to be exposed to quality insurance assessments with regular audits to create positive retroactive improvements of the system. Recommendations to the Ministry of Agriculture will be made to integrate this network into the National Research Centers Program to ensure a long-term financial sustainability. This continuity in the TDR monitoring is necessary to follow the evolution of the situation and to give to the farmers some guidelines for disease control that are provided as feedback from the samplings of their animals. The general conclusion that can be drawn from the first cycle of activity of the RESCAO (financed by the ITM/DGD framework agreement FAIII) is that in the RESCAO-area, TDR is widespread in *T. vivax* (most abundant trypanosome species) as well as in *T. congolense*.

After confirmation of the widespread presence of TDR in the field, the question was raised whether this was posing a significant problem for livestock breeding in that area. In other words, does a treatment failure unequivocally lead to a decrease in meat and milk production, to a poor body condition and even to the death of the animals as it would be logically expected?

Different factors should be here first reminded: (i) parasitaemia is commonly low in the field and might be under the sensitivity level of microscopy, (ii) trypanosome strains vary greatly in virulence, (iii) different levels of TDR are encountered and for some of the trypanosomes, trypanocidal drugs remain toxic even if not lethal. Therefore, the combination of classic microscopy and more sensitive molecular tools allowed to partially answering the above raised question. Indeed, when considering a positive microscopical diagnosis after a treatment with trypanocidal drugs, the relapsing animal is normally dying. This was the general observation of many field workers but like the invisible part of the iceberg many "hidden" things are happening that a microscope cannot detect. After treatment with trypanocidal drugs, some of the animals still present an active infection but at a microscopically undetectable parasite level (negative microscopic examination – sometimes PCR positive) that is compatible with an acceptable health status. Those low parasite levels probably allow the animals to control the less virulent trypanosomes even if they are drug resistant. Actually, some studies suggests that even in the presence of drug resistance, the continuing use of trypanocides (to a limited extent and in combination with other control measures) would allow the host to control the parasite and the corresponding disease at an acceptable physiological level (Rowlands et al., 1994; Geerts et al., 2001; Delespaux et al., 2010; Chitanga et al., 2011). In this case, TDR might be more a slight decrease in the sensitivity of trypanosome than a complete resistance against the toxic effects of the drug. In this context of low level of TDR (slight decrease of sensitivity to the drug), the deleterious effect of the disease might be less visible, especially in cattle infected by the less virulent trypanosome strains. Moreover, Gardiner (1989) showed that self-cure is relatively common in *T. vivax* infections and animals that recovered spontaneously from an acute infection developed chronic and asymptomatic infections (Batista et al., 2009). Therefore, the treatment with a trypanocide combined with the immune system of the host could increase this recovery rate. The cumulative effect of the drug and the immunity of the host might allow a better control of the parasites. This is resulting in very low oscillating parasitaemia with no or little impact on the host's health. The real concerns would thus be the highly virulent, highly resistant trypanosomes. Fortunately, those trypanosomes do not constitute at present the majority of the population, as demonstrated in the chapter 4 of this thesis. The evolution of the virulence in parallel

with drug resistance under continuous drug pressure constitutes an excellent and challenging subject for further research.

6.3. Molecular tools used to diagnose TDR in West Africa

Assessing the impact of TDR in livestock health and production, and analyzing the distribution of TDR in West Africa have been made easier by using molecular tools for the diagnosis of trypanosome infection and of DA resistance (for *T. congolense*). The newly improved molecular tool for the diagnosis of *T. congolense* DA-resistance is now an expertise of CIRDES, which is the sole West African Research Center, using routinely the *PCR-RFLP-Msp*I and *PCR-RFLP-Dpn*II tests for the diagnosis of AAT and DA resistance, respectively. However, further validation of the DA resistance test will be necessary after the recent observation that the target gene in *T. congolense* on which the test is based (*TcoAT1 / TcoNT10*) is not responsible for the transport of the drug (Munday et al., 2013). From a diagnostic point of view, this should not be a major issue in the case the detected mutation in the *TcoAT1 / TcoNT10* gene is linked to the genetic determinant responsible for the resistance phenotype elsewhere on the same chromosome. The data available so far strongly suggest this type of linkage for the *TcoAT1 / TcoNT10* gene (Delespaux and de Koning, 2013).

Another issue might be more problematic. We have seen that the mutation linked to DA resistance might be present in trypanosomes that disappear from the host (microscopical examination) following DA treatment but are not killed by the administration of 10 or even 20mg/kg DA in a mice test (Chitanga et al., 2011). When PCR is applied on the blood of such animals, low fluctuating parasitaemia are observed that are not detectable with the microscopical examination. In the mouse model, Chitanga et al. (2011) observed an increased relapse rate (detected by PCR) from 39% to 66.7% for 20 mg/kg simply by increasing the observation frequency from a single PCR-based detection at the end of the observation period (day 60) to weekly PCR-based parasite detections (8 in total), although all mice remained microscopically negative during the entire observation period. Exactly the same trend was observed in the goat experiment that we presented in the fourth chapter. These observations were the basis of our definition of TDR as “the decreased or absence of sensitivity of trypanosome strains to standard quality trypanocidal drugs at the dose recommended by the

manufacturer and administered according to good veterinary practices". We included in this definition those very low parasitaemia that are not affecting the health status of the animals. For a routine AAT diagnosis, the microscopic examination might be more appropriate and the molecular tool will be more useful for epidemiological surveys. The molecular tool will permit to have an overview of the situation of TDR in wide areas (e.g. West Africa region in the framework of the RESCAO), or to undertake TDR study in areas and/or in animals difficult to access (e.g. wild animals).

The situation with *T. vivax* is even more complex as no nucleoside transporter identified so far seems to be involved in DA resistance. After screening of all the nucleoside transporters available in the genomic database (www.genedb.org) by sequencing, no conserved polymorphism was observed between sensitive and resistant strains. Unfortunately, the sequence of one gene (TvY486_1103760) is incomplete and we cannot rule out that a conserved mutation linked to DA resistance might be located on this last unsequenced fragment. However, our study was based on a limited set of sequencing data and would advantageously be completed by whole genome sequencing on more than 4 characterized strains.

6.4. Managing animal trypanosomosis in West Africa

Africa faces the vital problem of feeding its people and one of the major challenge is to reduce the constraints on agriculture that constitute cross-border epizootics diseases such as animal trypanosomosis (Swallow, 1998; Shaw, 2003; Ilemobade, 2009). Indeed, African's traditional livestock owners are still considering animal trypanosomosis as the most significant threat to their cattle (Kone et al., 2012). Strategies for reducing the impact of trypanosomosis on animal health strongly depend on local factors such as fly pressure, infection rate of the flies, virulence of the trypanosomes, cattle breed, breeding conditions, availability of veterinary services, food for the cattle, drugs of good quality and of course the drug resistance situation (absence, presence, simple or multiple,…). Trypanocidal drugs are thus essential in the management of the disease but are not the sole factor. A "scale perspective" should be considered. Programs like the PATTEC aim at the complete eradication of the tsetse fly vector (Bouyer et al., 2013; Shaw et al., 2013). This is one possible option but is logistically heavy, very expensive and absolutely not proved to be possible at the continental scale. While waiting for this 'hypothetical' total

eradication of tsetse flies, farmers have to cope with the disease and drugs are one of the weapons at their disposition and mostly their first reflex. In most of the cases, farmers prefer treating their most productive animals (draft oxen, dairy cow, etc) and therefore do not hesitate to reduce the quantity of drugs provided to the other animals of the herd. Moreover, prevention is less obvious for farmers as most of them are breeding for many reasons (banking system, a bit of milk and manure, draught power, social significance, …) but not really for making a 'business' out of it (Affognon et al., 2009; Grace et al., 2009). They consider low or no input in the system. The lack of knowledge/education is focusing the management of the disease on drugs albeit many other options are possible when the relationship between the disease and the biology of the vector is understood. Common sense measures such as strategic watering of the cattle either by avoiding the early or late watering or by providing water at places where the flies are not present can already constitute an excellent prevention method. Other options are available such as restricted grazing, insecticide impregnated nets, localized spraying (belly and legs). However, these measures demand knowledge (Bouyer et al., 2007; Bouyer et al., 2009). Even the nature of the traditional breeding system by itself constitutes a major pitfall. What is the aim of maintaining animals for more than 10 years as it is often the case? The period of exposition to the vector is extremely long and meanwhile the cattle are fed with poor or no production in exchange. This constitutes a major threat to the environment and may represent an economic non-sense. A first priority in an "ideal world" would be to limit the breeding to the productive phase and to grab the benefits when present i.e. to transform the meat in currency and bank it. This demand political and financial stability. The use of drugs in this scenario would be drastically decreased. However as traditions are very difficult to change, farmers will still have to cope with drugs and drug resistance. Knowing that and the fact that TDR is a reality, concentrating efforts on the development of new drugs remains a realistic issue. It is still too often stated that pharmaceutical companies are not keen on developing new drugs because of the "limited" African market (Sones, 2001; Affognon, 2007). This is a kind of dogmatic assertion as the disease also affect large parts of South America (Batista et al., 2007; Batista et al., 2009), China (Lun et al., 1993; Tang et al., 2012) and the Indian subcontinent (Ravindran et al., 2008; Desquesnes et al., 2013). What could thus be an ideal drug in such a context? The following characteristics should be considered: (i) efficient against all the pathogenic trypanosome species to allow for a "global market",

(ii) good quality (good stability when diluted, etc), (iii) single dose packaging to avoid under dosage, (iv) different from sleeping sickness drugs to prevent the development of cross-resistance, (v) widely available and (vi) at a reasonable price. In the framework of the GALVmed project (Global Alliance for Livestock Veterinary Medicine - http://www.galvmed.org), new medications were tested against drug resistant, highly virulent trypanosome isolates originating from Cameroon. Cattle inoculated with those strains and treated with those new compounds were effectively cured and completely cleared of the parasites. Further toxicological studies are ongoing. This brings hope for some hot spots of drug resistance (Delespaux, pers. Comm.).

In the Chapter 5 of this PhD thesis, we discussed about an alternative consisting in potentiating the efficacy of ISM by associating it to two veterinary affordable antibiotics drugs, i.e. oxytetracycline and enrofloxacin. Our results were encouraging even if some factors should have been further explored like the best galenic formula, the optimal combination of potentiator with ISM and the linking of the potentiator to nanobodies directed against the trypanosomes for a rapid internalization of the compound. Different attempts were made to find some pharmaceutical companies willing to contribute financially to the research but without success so far. While waiting for new products on the market, the Rational Drug Use method described by Clausen et al. (2010) is currently the coherent approach to adopt. It permits to better control all the chain of drug administration, from purchasing to injecting the drug (Clausen et al., 2010; Mungube et al., 2012).

Considering existing and potential new drugs, the reinforcement and the harmonization of veterinary legislation and proper quality control of the trypanocidal drugs is the corner stone of the effective treatments of the animals. This reinforcement started in 2006 in the "Union Economique et Monétaire Ouest-Africaine" (UEMOA) zone with the deliverance of a unique marketing authorization (MA) to any pharmaceutical company that commercialize its products. This will certainly contribute to reduce the problem of TDR (Daré, 2007). However, more focused actions are necessary for the control of veterinary drugs sold at the local markets (Figure 6.1). In the framework of a collaboration FAO – GALVmed – TRYRAC (Trypanosomosis Rational Chemotherapy - http://www.trypanocide.eu/), it is now possible for any private person or institution to

check the quality of different medications including trypanocides for a cost of 300€/molecule (LACOMEV – Dakar – Senegal -http://www.eismv.org/Jumelage-entre-l-Agence-Nationale.html). This is a great step forward as the threat is now present for pharmaceutical companies to be tested and openly banned from the market.

Figure 6.1: Veterinary drugs in a West African market (Affognon et al., 2009).

6.5. Conclusion

This thesis should be considered as a small piece being part of a much larger edifice, which is the impact of trypanosomosis on the livelihood of farmers. I tried to bring a bit of light in the comprehension of this problematic by considering (i) the diagnosis of drug resistance, paramount parameter in the management of drug resistance, (ii) the human adventure of coordinating the activities of eight African countries in a same ideal, which will be necessary for the resolution of the problem in a mid or long term perspective, (iii) the real impact of drug resistance on the health status of infected animals, which will be of uttermost importance in the future (whether it's a new fine-tuned balance between host and parasite with compromises from both parts or not) and (iv) the use of low-cost old means that combined together can bring new insights on handling the

disease. This thesis work caused in my brain the widest admiration for a small fly and a microscopic parasite that are challenging a large population of international researchers for more than a century. However, I was really pleased to humbly participate to some minor moves of this gigantic chess game.

6.6. Reference List

Affognon, H., 2007. Economic analysis of trypanocide use in villages under risk of drug resistance in West Africa. PhD thesis. In. Gottfried Wilhelm Leibniz University of Hannover, Germany. 195p.

Affognon, H., Coulibaly, M., Diall, O., Grace, D., Randolph, T., Waibel, H., 2009. Gestion de la chimiorésistance dans le cadre de la lutte intégrée contre la Trypanosomose dans la zone cotonnière de l'Afrique de l'Ouest. In. ILRI Research Report, N°17, Nairobi, Kenya. 58p.

Authie, E., 1984. Mise en évidence d'une résistance aux trypanocides parmi les souches de *Trypanosoma congolense* récemment isolés au Burkina Faso. Rev. Elev. Méd. Vét. Pays trop. 37, 219-235.

Batista, J.S., Oliveira, A.F., Rodrigues, C.M.F., Damasceno, C.A.R., Oliveira, I.R.S., Alves, H.M., Paiva, E.S., Brito, P.D., Medeiros, J.M.F., Rodrigues, A.C., Teixeira, M.M.G., 2009. Infection by *Trypanosoma vivax* in goats and sheep in the Brazilian semiarid region: From acute disease outbreak to chronic cryptic infection. Vet. Parasitol. 165, 131-135.

Batista, J.S., Riet-Correa, F., Teixeira, M.M., Madruga, C.R., Simoes, S.D., Maia, T.F., 2007. Trypanosomiasis by *Trypanosoma vivax* in cattle in the Brazilian semiarid: Description of an outbreak and lesions in the nervous system. Vet. Parasitol. 143, 174-181.

Bouyer, J., Seck, M.T., Sall, B., 2013. Misleading guidance for decision making on tsetse eradication: Response to Shaw et al. (2013). Prev. Vet. Med. http://dx.doi.org/10.1016/j.prevetmed.2013.05.017.

Bouyer, J., Stachurski, F., Gouro, A.S., Lancelot, R., 2009. Control of bovine trypanosomosis by restricted application of insecticides to cattle using footbaths. Vet. Parasitol. 161, 187-193.

Bouyer, J., Stachurski, F., Kabore, I., Bauer, B., Lancelot, R., 2007. Tsetse control in cattle from pyrethroid footbaths. Prev. Vet. Med. 78, 223-238.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J., Delespaux, V., 2011. High Prevalence of Drug Resistance in Animal Trypanosomes

without a History of Drug Exposure. PLoS Negl. Trop. Dis. 5 (12): e1454. doi:10.1371/journal.pntd.0001454.

Clausen, P.H., Bauer, B., Zessin, K.H., Diall, O., Bocoum, Z., Sidibe, I., Affognon, H., Waibel, H., Grace, D., Randolph, T., 2010. Preventing and containing trypanocide resistance in the cotton zone of West Africa. Transbound. Emerg. Dis. 57, 28-32.

Daré, I., 2007. Harmonisation de l'enrégistrement et du contrôle des médicaments vétérinaires en Afrique: L'exemple de l'Union Economique et Monétaire Ouest-Africaine (UEMOA). In. Conf. OIE 2007, pp. 133-147.

Delespaux, V., de Koning, H.P., 2013. Transporters in Anti-Parasitic Drug Development and Resistance. In: Jäger, T., Och, O., Flohé, L. (Eds.), Trypanosomatid Diseases: Molecular Routes to Drug Discovery. Published by Wiley-VCH Verlag GmbH & Co. KGaA, pp. 335-351.

Delespaux, V., Vitouley, S.H., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Geerts, S., Van den Bossche, P., 2010. Chemosensitization of *Trypanosoma congolense* strains resistant to isometamidium chloride by tetracycline and enrofloxacin. PLoS Negl. Trop Dis. 4, 2-8.

Desquesnes, M., Holzmuller, P., Lai, D.H., Dargantes, A., Lun, Z.R., Jittaplapong, S., 2013. *Trypanosoma evansi* and Surra: A Review and Perspectives on Origin, History, Distribution, Taxonomy, Morphology, Hosts, and Pathogenic Effects. Biomed. Res. Int. Volume 2013, Article ID 194176, 22 pages. http://dx.doi.org/10.1155/2013/194176.

Gardiner, P.R., 1989. Recent studies of the biology of *Trypanosoma vivax*. Adv. Parasitol. 28, 229-317.

Geerts, S., Holmes, P.H., Diall, O., Eisler, M.C., 2001. African bovine trypanosomiasis: the problem of drug resistance. Trends Parasitol. 17, 25-28.

Grace, D., 2005. Epidemiology and control of cattle trypanosomosis in villages under risk of trypanocide resistance in West Africa. PhD thesis. In. Institute for Parasitology and International Animal Health, Free University of Berlin, Germany. 201p.

Grace, D., Randolph, T., Affognon, H., Dramane, D., Diall, O., Clausen, P.H., 2009. Characterisation and validation of farmers' knowledge and practice of cattle trypanosomosis management in the cotton zone of West Africa. Acta Trop. 111, 137-143.

Ilemobade, A.A., 2009. Tsetse and trypanosomosis in Africa: the challenges, the opportunities. Onderstepoort J. Vet. Res. 76, 35-40.

Kone, N., Bouyer, F., Vitouley, H.S., Sidibe, I., N'Goran, E.K., Vial, L., Balenghien, T., Bouyer, J., 2012. Perceptions des éleveurs et stratégies de gestion du risque trypanosomien dans le bassin du fleuve Mouhoun (Burkina Faso). Cah. Agric. 21: 404-16. doi: 10.1684/agr.2012.0599.

Lun, Z.R., Fang, Y., Wang, C.J., Brun, R., 1993. Trypanosomiasis of domestic animals in China. Parasitol. Today 9, 41-45.

McDermott, J., Woitag, T., Sidibe, I., Bauer, B., Diarra, B., Ouedraogo, D., Kamuanga, M., Peregrine, A.S., Eisler, M.C., Zessin, K.H., Mehlitz, D., Clausen, P.H., 2003. Field studies of drug-resistant cattle trypanosomes in Kenedougou Province, Burkina Faso. Acta Trop. 86, 93-103.

Munday, J.C., Rojas Lopez, K.E., Eze, A.A., Delespaux, V., Van Den Abbeele, J., Rowan, T., Barrett, M.P., Morrison, L.J., de Koning, H.P., 2013. Functional expression of *TcoAT1* reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake. International Journal for Parasitology: Drugs and drug resistance 3, 69-76.

Mungube, E.O., Diall, O., Baumann, M.P., Hoppenheit, A., Hinney, B., Bauer, B., Sanogo, Y., Maiga, B., Zessin, K.H., Randolph, T.F., Clausen, P.H., 2012. Best-bet integrated strategies for containing drug-resistant trypanosomes in cattle. Parasit. Vectors. 5, 164.

Ravindran, R., Rao, J.R., Mishra, A.K., Pathak, K.M.L., Babu, N., Satheesh, C.C., Rahul, S., 2008. *Trypanosoma evansi* in camels, donkeys and dogs in India:comparison of PCR and light microscopy for detection. Veterinarski Arhiv 78, 89-94.

Rowlands, G.J., Mulatu, W., Authie, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M., 1994. Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug resistant trypanosomes. Prev. Vet. Med. 21, 87-101.

Shaw, A.P.M., 2003. Economic guidelines for strategic planning of tsetse and trypanosomiasis control in West Africa. Food & Agriculture Organization - 75p, Rome.

Shaw, A.P., Torr, S.J., Waiswa, C., Cecchi, G., Wint, G.R., Mattioli, R.C., Robinson, T.P., 2013. Estimating the costs of tsetse control options: an example for Uganda. Prev. Vet. Med. 110, 290-303.

Sones, K., 2001. Pharmaceutical companies: partners or enemies? ICPTV Newsletter 3, 19-21.

Swallow, B.M., 1998. Impacts of African animal trypanosomosis on migration, livestock and crop production. PAAT Position Paper, FAO-OMS-IAEA-OAU/IBAR, 1-19.

Talaki, E., 2008. Etude de la résistance des trypanosomes à l'isométamidium et au diminazène dans la zone cotonnière de l'Afrique de l'Ouest (Mali - Guinée - Burkina Faso). PhD thesis. In. Université Polytechnique de Bobo-Dioulasso / Institut de Développement Rural / Département d'Elevage, pp. 1-160.

Tang, H.J., Lan, Y.G., Wen, Y.Z., Zhang, X.C., Desquesnes, M., Yang, T.B., Hide, G., Lun, Z.R., 2012. Detection of *Trypanosoma lewisi* from wild rats in Southern China and its genetic diversity based on the ITS1 and ITS2 sequences. Infect. Genet. Evol. 12, 1046-1051.

SUMMARY

For more than half a century, livestock trypanosomosis in West Africa has been the subject of various research papers published in the scientific literature. Since the discovery of the first *T. congolense* resistant strain in 1984, several studies have alerted on the increasing phenomenon of trypanocidal drug resistance (TDR) in the cotton belt of West Africa against the two commonly molecules used in this area i.e. diminazene aceturate and isometamidium chloride. The recently developed molecular tools for the diagnosis of TDR allowed gaining better information on the spread of TDR. The work that is presented in this thesis aimed at (i) improving the performance of the PCR-RFLP tool used for the diagnosis of diminazene aceturate resistance in *T. congolense* under field conditions, (ii) prospect the development of a similar molecular tool for the diagnosis of *T. vivax* resistance to diminazene aceturate, and using these molecular tools to monitor TDR in West Africa through the epidemiosurveillance network of chemoresistance to trypanocidal and acaricides drugs in West Africa called RESCAO, (iii) determining the impact of TDR on livestock health and production in endemic areas of West Africa and (iv) proposing an alternative to contain or reduce the TDR phenomenon.

The first chapter of this thesis gives general information on the disease and its components (parasite and vector). Moreover, it reviews our current knowledge on the pharmacology of diminazene aceturate, isometamidium chloride and homidium salts and on the phenomenon of trypanocidal drug resistance.

Chapter 2 describes the work for the improvement of the performance of a PCR-RFLP for the detection of diminazene resistance in *T. congolense* under field conditions. We used blood spots on filters papers collected from parasitologically positive cattle in South-east Mali. This study permitted to increase the specificity of the PCR-RFLP test and to enhance its sensitivity in determining a low parasitaemia observed in the field. This molecular tool was used to update the current situation of *T. congolense* resistance to diminazene aceturate in the countries that are members of RESCAO - the West African's epidemiosurveillance network of chemoresistance to trypanocidal and

acaricides drugs. From this study we can learn that TDR in widely distributed West Africa.

In chapter 3, an exhaustive exploration of the genes predicted to code for nucleoside transporters in *T. vivax* was performed. For this analysis, *T. vivax* samples used were already characterized in goats for their sensitivity and/or resistance against diminazene aceturate. SSCP analysis and sequencing shows that the P2-type putative adenosine transporters genes seem not to been involved in diminazene resistance in this trypanosome species. More studies are therefore needed to understand this mechanism of resistance or alternatively to find a trustable genetic marker.

Chapter 4 aimed at studying the impact of TDR in livestock health and production in endemic areas of West Africa. We used 60 female goats infected with *T. vivax* strains collected in the vicinity of Bobo-Dioulasso (Burkina Faso). This study showed that drug resistant *T. vivax* strains inoculated to goats might probably had no significant impact on the PCV and body weight losses except for a few highly virulent strains. This still needs to be confirmed in cattle under natural tsetse and *Trypanosoma* challenge.

Chapter 5 aimed at finding an alternative to contain and/or reduce TDR by potentiating the efficacy of isometamidium chloride by associating it to two veterinary affordable antibiotics i.e. tetracyclines and enrofloxacin. The obtained results are encouraging even if more studies are still needed to determine the best galenic solution, the optimal combination of the chemosensitizer with isometamidium and to test this combination in livestock under controlled and field conditions in areas with high tsetse challenge and high trypanocidal drug resistance.

In the last chapter (Chapter 6), the major findings of the thesis are discussed in the wide context of the management of animal trypanosomosis in West Africa.

SAMENVATTING

Reeds meer dan een halve eeuw, is trypanosomose bij het vee in West-Afrika het onderwerp van verschillende onderzoeken gepubliceerd in de wetenschappelijke literatuur. Sinds de ontdekking van de eerste *T. congolense* trypanocide resistente stam in 1984, toonden verschillende studies een toename van het probleem van trypanocide resistentie (TDR) –tegen diminazene en isometamidium - in de "Cotton belt" van West Afrika. De recent ontwikkelde moleculaire methoden voor de diagnose van TDR hebben bijgedragen tot een beter inzicht van de epidemiologie.

Het onderzoek dat deel uitmaakt van dit proefschrift is gericht op (i) het verbeteren van de PCR-RFLP methode voor de diagnose van diminazene resistentie van *T. congolense* in veldomstandigheden, (ii) het ontwikkelen van een soortgelijke moleculaire methode voor de diagnose van *T. vivax* resistentie tegen diminazene, (iii) het bestuderen, met de hierboven moleculaire methoden, van TDR in West-Afrika door middel van het epidemiologische netwerk van chemoresistentie tegen trypanocide en acaricide middelen in West Afrika, genaamd RESCAO, (iv) het bepalen van de impact van TDR voor de dierlijke gezondheid en productie in endemische gebieden van West Afrika en tenslotte (v), het uittesten van alternatieve behandelingsmethoden om TDR te beperken.

Het eerste hoofdstuk vat onze huidige kennis samen over de dierlijke trypanosomen en hun vectoren, de pathogenese van trypanosomosis, de diagnose, behandeling en controle; en gaat dieper in op de farmacologie van diminazene aceturate, isometamidium chloride en homidium zouten en trypanocide resistentie.

In hoofdstuk 2 wordt een studie beschreven naar de verbetering van de gevoeligheid en de specificiteit van een PCR-RFLP onder veldomstandigheden voor de detectie van resistentie bij *T. congolense* tegen diminazene. Er werd gebruik gemaakt van de verbeterde moleculaire methode om *T. congolense* resistentie tegen diminazene op te volgen in de landen leden van RESCAO, de west Afrikaanse netwerk van epidemio-bewaking van trypanocide en acaricide resistentie. Dit onderzoek toonde een uitgebreide verspreiding aan van TDR in West Afrikaanse landen die lid zijn van de RESCAO.

In hoofdstuk 3 wordt een uitgebreide screening van *nucleoside transporters* beschreven bij *T. vivax*. Er werd gezocht naar geconserveerde sequentie veranderingen in resistente fenotypes. Daarvoor werd gebruik gemaakt van *T. vivax* monsters die reeds gekarakteriseerd waren voor hun gevoeligheid en / of resistentie tegen diminazene. Uit de SSCP analyse en sequentiebepaling bleek dat de P2-type *adenosine transporter* genen geen rol spelen in diminazene resistentie bij *T. vivax* zoals het waarschijnlijk wel het geval is voor *T. congolense*. Uitgebreider onderzoek is daarom noodzakelijk om dit mechanisme van resistentie te begrijpen of om een betrouwbaar genetisch merker van diminazene resistente te vinden bij *T. vivax*.

Hoofdstuk 4 was gericht op het bestuderen van de impact van TDR op de gezondheid en productie van geiten in endemische gebieden van West-Afrika. Zestig vrouwelijke geiten werden experimenteel besmet met *T. vivax* stammen verzameld in de omgeving van Bobo-Dioulasso (Burkina Faso). Deze studie toonde aan dat resistente *T. vivax* stammen geënt in geiten geen significante invloed hadden op de PCV en het lichaamsgewicht, behalve voor een paar zeer virulente stammen. Dit is in tegenspraak met de algemene opinie dat de ontwikkeling van resistentie tegen trypanociden aan de boeren geen uitkomst zou laten.

In hoofdstuk 5 werd gezocht naar een alternatieve behandeling voor het beperken van TDR door het samen gebruiken van isometamidium met twee in de diergeneeskunde courant gebruikte en betaalbare antibiotica: tetracyclines en enrofloxacine. De preliminaire resultaten waren bemoedigend. Toch is uitgebreider onderzoek noodzakelijk om de beste galenische oplossing uit te werken, de optimale combinatie van de antibiotica met isometamidium te bepalen en deze combinatie te testen op dieren onder gecontroleerde omstandigheden en in veldcondities, in gebieden met hoge tsetse druk en met belangrijke trypanocide resistentie.

In het laatste hoofdstuk (hoofdstuk 6), werden de belangrijkste bevindingen van dit proefschrift besproken in het brede kader van de epidemiologie van trypanocide resistentie in West-Afrika.

Annex

The exploration of the nucleoside transporter TvY486_0014570 in sequencing show no conserved polymorphism between the sensitive and resistant *T. vivax* strains used (see below).

```
>TvY486_14570F1        TCCTGTCTTCACACTATTAGACAAGC
>TvY486_14570R1        GAGAAGTATATGTACGACTGGGTCAA

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           TCCTGTCTTCACACTATTAGACAAGCTAGAATCTTAACAACATCAAGTTCATTCCGCGGC
H89_resistant           TCCTGTCTTCACACTATTAGACAAGCTAGAATCTTAACAACATCAAGTTCATTCCGCGGC
H988_sensitive          TCCTGTCTTCACACTATTAGACAAGCTAGAATCTTAACAACATCAAGTTCATTCCGCGGC
H1008_sensitive         TCCTGTCTTCACACTATTAGACAAGCTAGAATCTTAACAACATCAAGTTCATTCCGCGGC
ILRAD700_sensitive      TCCTGTCTTCACACTATTAGACAAGCTAGAATCTTAACAACATCAAGTTCATTCCGCGGC
                        ************************************************************

H65_resistant           GAGATGGGGTTGCTGGGTTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTC
H89_resistant           GAGATGGGGTTGCTGGGTTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTC
H988_sensitive          GAGATGGGGTTGCTGGGTTTTGAGTCGCCAGCGGCCTTTGTCGTCTATTTGAGCTTCCTC
H1008_sensitive         GAGATGGGGTTGCTGGGTTTTGAGTCGCCAGCGGCCTTTGTCGTCTATTTGAGCTTCCTC
ILRAD700_sensitive      GAGATGGGGTTGCTGGGTTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTC
                        ********************************** ************************

H65_resistant           TCGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTATTGCTTGTATGAATATTTT
H89_resistant           TCGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTATTGCTTGTATGAATATTTT
H988_sensitive          TCGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTATTGCTTATATGAATACTTT
H1008_sensitive         TCGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTATTGCTTATATGAATACTTT
ILRAD700_sensitive      TCGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTATTGCTTGTATGAATATTTT
                        ********************************************** ******** ***

H65_resistant           ACACAGTTCTTCAAACTTGCCCAGGGAAAGTCGGACGTCGACCCTGCGGACGCCGCGTTC
H89_resistant           ACACAGTTCTTCAAACTTGCCCAGGGAAAGTCGGACGTCGACCCTGCGGACGCCGCGTTC
H988_sensitive          ACACAGTTCTTCAAACTTGCCCAGGGAAAGTCGGACGTCGACCCTGCGGACGCCGCGTTC
H1008_sensitive         ACACAGTTCTTCAAACTTGCCCAGGGAAAGTCGGACGTCGACCCTGCGGACGCCGCGTTC
ILRAD700_sensitive      ACACAGTTCTTCAAACTTGCCCAGGGAAAGTCGGACGTCGACCCTGCGGACGCCGCGTTC
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H65_resistant          TGGACGAACATTCACACGTACTACAACGTGACTGTCTTTTCAACGCAGGTTGTTGCCGAG
H89_resistant          TGGACGAACATTCACACGTACTACAACGTGACTGTCTTTTCAACGCAGGTTGTTGCCGAG
H988_sensitive         TGGACGAACATTCACACGTACTACAACGTGACTGTCTTTTCAACGCAGGTTGTTGCCGAG
H1008_sensitive        TGGACGAACATTCACACGTACTACAACGTGACTGTCTTTTCAACGCAGGTTGTTGCCGAG
ILRAD700_sensitive     TGGACGAACATTCACACGTACTACAACGTGACTGTCTTTTCAACGCAGGTTGTTGCCGAG
                       ************************************************************

H65_resistant          ATATTCATGCTGACGCCACTTGGTAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGCT
H89_resistant          ATATTCATGCTGACGCCACTTGGTAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGCT
H988_sensitive         ATATTCATGCTGACGCCACTTGGTAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGCT
H1008_sensitive        ATATTCATGCTGACGCCACTTGGTAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGCT
ILRAD700_sensitive     ATATTCATGCTGACGCCACTTGGTAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGCT
                       ************************************************************

H65_resistant          TTTGCCCTGCCGTTTTTGCAACTCTCTTCGTACATGCTGACCACCACGTTCCATACAACG
H89_resistant          TTTGCCCTGCCGTTTTTGCAACTCTCTTCGTACATGCTGACCACCACGTTCCATACAACG
H988_sensitive         TTTGCCCTGCCGTTTTTGCAACTCTCTTCGTACATGCTGACCACCACGTTTCATACAACG
H1008_sensitive        TTTGCCCTGCCGTTTTTGCAACTCTCTTCGTACATGCTGACCACCACGTTCCATACAACG
ILRAD700_sensitive     TTTGCCCTGCCGTTTTTGCAACTCTCTTCGTACATGCTGACCACCACGTTCCATACAACG
                       ************************************************** *********

H65_resistant          GAAAACGGTGCAAAAGCCTTATTCCTGGTCATGGCATTTGTGAACGGCCTGTCAAAGTCA
H89_resistant          GAAAACGGTGCAAAAGCCTTATTCCTGGTCATGGCATTTGTGAACGGCCTGTCAAAGTCA
H988_sensitive         GAAAACGGTGCAAAAGCCTTATTCCTGGTCATGGCATTTGTGAACGGCCTGTCAAAGTCA
H1008_sensitive        GAAAACGGTGCAAAAGCCTTATTCCTGGTCATGGCATTTGTGAACGGCCTGTCAAAGTCA
ILRAD700_sensitive     GAAAACGGTGCAAAAGCCTTATTCCTGGTCATGGCATTTGTGAACGGCCTGTCAAAGTCA
                       ************************************************************

H65_resistant          TTCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCA
H89_resistant          TTCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCA
H988_sensitive         TTCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCA
H1008_sensitive        TTCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCA
ILRAD700_sensitive     TTCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCA
                       ************************************************************

H65_resistant          TACGTCTTTGGTCTTCCACTTTCTGGCGTCATCACTGCCATTCTGTCCTTGGTGATCAAG
H89_resistant          TACGTCTTTGGTCTTCCACTTTCTGGCGTCATCACTGCCATTCTGTCCTTGGTGATCAAG
H988_sensitive         TACGTCTTTGGACTCCCACTTTCTGGCGTCATCACTGCCATTCTGTCCTTGGTGATCAAG
H1008_sensitive        TACGTCTTTGGACTCCCACTTTCTGGCGTCATCACTGCCATTCTGTCCTTGGTGATCAAG
ILRAD700_sensitive     TACGTCTTTGGTCTTCCACTTTCTGGCGTCATCACTGCCATTCTGTCCTTGGTGATCAAG
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H65_resistant           GGGTCAATGAACAACGACTTTGACAATCTCTTGACCCAGTCGTACATATACTTCTC
H89_resistant           GGGTCAATGAACAACGACTTTGACAATCTCTTGACCCAGTCGTACATATACTTCTC
H988_sensitive          GGGTCAATGAACAACGACTTTGACAATCTCTTGACCCAGTCGTACATATACTTCTC
H1008_sensitive         GGGTCAATGAACAACGACTTTGACAATCTCTTGACCCAGTCGTACATATACTTCTC
ILRAD700_sensitive      GGGTCAATGAACAACGACTTTGACAATCTCTTGACCCAGTCGTACATATACTTCTC
                        ************************************************************


>TvY486_14570F2         CTCTTGACCCAGTCGTACATATACTT
>TvY486_14570R2         GTTGTCCTTTATCACCTTCTGTTGAC

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           CTCTTGACCCAGTCGTACATATACTTCTCCACGACAATGGCTTTTCAGGCCATCGCATGC
H89_resistant           CTCTTGACCCAGTCGTACATATACTTCTCCACGACAATGGCTTTTCAGGCCATCGCATGC
H988_sensitive          CTCTTGACCCAGTCGTACATATACTTCTCCACGACAATGGCTTTTCAGGCCATCGCATGC
H1008_sensitive         CTCTTGACCCAGTCGTACATATACTTCTCCACGACAATGGCTTTTCAGGCCATCGCATGC
ILRAD700_sensitive      CTCTTGACCCAGTCGTACATATACTTCTCCACGACAATGGCTTTTCAGGCCATCGCATGC
                        ************************************************************

H65_resistant           GTTTTGTTGTACCTGCTCCCCAAGAATCCGTACGCCCTGCGGTACGCGGCGGAGCTCAGG
H89_resistant           GTTTTGTTGTACCTGCTCCCCAAGAATCCGTACGCCCTGCGGTACGCGGCGGAGCTCAGG
H988_sensitive          GTTTTGTTGTACCTGCTCCCCAAGAATCCGTACGCCCTGCGGTACGCGGCGGAGCTCAGG
H1008_sensitive         GTTTTGTTGTACCTGCTCCCCAAGAATCCGTACGCCCTGCGGTACGCGGCGGAGCTCAGG
ILRAD700_sensitive      GTTTTGTTGTACCTGCTCCCCAAGAATCCGTACGCCCTGCGGTACGCGGCGGAGCTCAGG
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H65_resistant           TACGCTATGAGGAAGAACAGCGCAGGTGGGAATGGAGGACACAGCACAAGAGACACAGAC
H89_resistant           TACGCTGTGAGGAAGAACAGCGCAGGTGGGAATGGAGGACACAACACAAGAGACACAGAC
H988_sensitive          TACGCTATGAGGAAGAACAGCGCAGGTGGGAATGGAGGACACAGCACAAGAGACACAGAC
H1008_sensitive         TACGCTATGAGGAAGAACAGCGCAGGTGGGAATGGAGGACACAGCACAAGAGACACAGAC
ILRAD700_sensitive      TACGCTATGAGGAAGAACAGCGCAGGTGGGAATGGAGGACACAGCACAAGAGACACAGAC
                        ****** ********************************** ****************

H65_resistant           AGACCCGAATCCCTCCTAACAGGCGAGCCTGTGAATGACGGTGACCAAGCACAGCCGGTG
H89_resistant           AGACCCGAATCCCTCCTAACAGGCGAGCCTGTGAATGACGGTGACCAAGCACAGCCGGTG
H988_sensitive          AGACCCGAATCCCTCCTAACAGGCGAGCCTGTGAATGACGGTGACCAAGCACAGCCGGTG
H1008_sensitive         AGACCCGAATCCCTCCTAACAGGCGAGCCTGTGAATGACGGTGACCAAGCACAGCCGGTG
ILRAD700_sensitive      AGACCCGAATCCCTCCTAACAGGCGAGCCTGTGAATGACGGTGACCAAGCACAGCCGGTG
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H65_resistant          GTGAGGAGCGTCCTGGACACGACAGTTGATCCGGATACGATGAAGGACACTGATCAGGTG
H89_resistant          GTGAGGAGCGTCCTGGACACGACAGTTGATCCGGATACGATGAAGGACACTGATCAGGTG
H988_sensitive         GTGAGGAGCGTCCTGGACACGACAGTTGATCCGGATACGATGAAGGACACTGATCAGGTG
H1008_sensitive        GTGAGGAGCGTCCTGGACACGACAGTTGATCCGGATACGATGAAGGACACTGATCAGGTG
ILRAD700_sensitive     GTGAGGAGCGTCCTGGACACGACAGTTGATCCGGATACGATGAAGGACACTGATCAGGTG
                       ************************************************************

H65_resistant          GAGGACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGTCGCCAAACGC
H89_resistant          GAGGACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGTCGCCAAACGC
H988_sensitive         GAGGACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGTCGCCAAACGC
H1008_sensitive        GAGGACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGTCGCCAAACGC
ILRAD700_sensitive     GAGGACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGTCGCCAAACGC
                       ************************************************************

H65_resistant          ATATACCCGATCCTCGCCACGTGCTTTTTCATCTACTTCTCAACGCTCCTCTTCTGGCCC
H89_resistant          ATATACCCGATCCTCGCCACGTGCTTTTTCATCTACTTCTCAACGCTCCTCTTCTGGCCC
H988_sensitive         ATATACCCGATCCTCGCCACGTGCTTTTTCATCTACTTCTCAACGCTCCTCTTCTGGCCC
H1008_sensitive        ATATACCCGATCCTCGCCACGTGCTTTTTCATCTACTTCTCAACGCTCCTCTTCTGGCCC
ILRAD700_sensitive     ATATACCCGATCCTCGCCACGTGCTTTTTCATCTACTTCTCAACGCTCCTCTTCTGGCCC
                       ************************************************************

H65_resistant          GGCGTCTTCATTGCTGTGGACTCACAAGGGTGGAACTTCTGGTACGGCACGATCGTGATG
H89_resistant          GGCGTCTTCATTGCTGTGGACTCACAAGGGTGGAACTTCTGGTACGGCACGATCGTGATG
H988_sensitive         GGCGTCTTCATTGCTGCGGACTCACAAGGGTGGAACTTCTGGTACGGCACGATCGTGATG
H1008_sensitive        GGCGTCTTCATTGCTGTGGACTCACAAGGGTGGAACTTCTGGTACGGCACGATCGTGATG
ILRAD700_sensitive     GGCGTCTTCATTGCTGTGGACTCACAAGGGTGGAACTTCTGGTACGGCACGATCGTGATG
                       *************** ****************************************

H65_resistant          GCTATGTTCAACTTTGGTGACTTCTTCTCGCGCTTGCAGCTTCAGTTCAAGAACCTCCAC
H89_resistant          GCTATGTTCAACTTTGGTGACTTCTTCTCGCGCTTGCAGCTTCAGTTCAAGAACCTCCAC
H988_sensitive         GCTATGTTCAACTTTGGTGACTTCTTCTCGCGCTTGCAGCTTCAGTTCAAGAACCTCCAC
H1008_sensitive        GCTATGTTCAACTTTGGTGACTTCTTCTCGCGCTTGCAGCTTCAGTTCAAGAACCTCCAC
ILRAD700_sensitive     GCTATGTTCAACTTTGGTGACTTCTTCTCGCGCTTGCAGCTTCAGTTCAAGAACCTCCAC
                       ************************************************************

H65_resistant          CCATCCCCGCGGACGGTGATCATCGCCGCGTTCGCTCGCCTGCTGATCATCGTGCCGCTC
H89_resistant          CCATCCCCGCGGACGGTGATCATCGCCGCGTTCGCTCGCCTGCTGATCATCGTGCCGCTC
H988_sensitive         CCATCCCCGCGGACGGTGATCATCGCCGCGTTCGCTCGCCTGCTGATCATCGTGCCGCTC
H1008_sensitive        CCATCCCCGCGGACGGTGATCATCGCCGCGTTCGCTCGCCTGCTGATCATCGTGCCGCTC
ILRAD700_sensitive     CCATCCCCGCGGACGGTGATCATCGCCGCGTTCGCTCGCCTGCTGATCATCGTGCCGCTC
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H65_resistant           TTCCTCTGTCAACAGAAGGTGATAAAGGACAAC
H89_resistant           TTCCTCTGTCAACAGAAGGTGATAAAGGACAAC
H988_sensitive          TTCCTCTGTCAACAGAAGGTGATAAAGGACAAC
H1008_sensitive         TTCCTCTGTCAACAGAAGGTGATAAAGGACAAC
ILRAD700_sensitive      TTCCTCTGTCAACAGAAGGTGATAAAGGACAAC
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Sequencing performed on TvY486_0043680 using 2 resistant and 3 sensitive *T. vivax* strains

```
>TvY486_0043680F1       aggtgcgctctcaactgaat
>TvY486_0043680R1       TTTGGACTCCCACTTTCTGG
```

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CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           AGGTGCGCTCTCAACTGAATTCCCCCATTTGATATCACCACGTGTACCAACCACTGTCCA
H89_resistant           AGGTGCGCTCTCAACTGAATTCCCCCATTTGATATCACCACGTGTACCAACCACTGTCCA
H988_sensitive          AGGTGCGCTCTCAACTGAATTCCCCCATTTGATATCACCACGTGTACCAACCACTGTCCA
H1008_sensitive         AGGTGCGCTCTCAACTGAATTCCCCCATTTGATATCACCACGTGTACCAACCACTGTCCA
ILRAD700_sensitive      AGGTGCGCTCTCAACTGAATTCCCCCATTTGATATCACCACGTGTACCAACCACTGTCCA
                        ************************************************************

H65_resistant           CTCTGTTTCACATTGTTAGAAAAGGAGCGATTTCGGCATCACTAAGTTCGTTCTACAGCA
H89_resistant           CTCTGTTTCACATTGTTAGAAAAGGAGCGATTTCGGCATCACTAAGTTCGTTCTACAGCA
H988_sensitive          CTCTGTTTCACATTGTTAGAAAAGGAGCGATTTCGGCATCACTAAGTTCGTTCTACAGCA
H1008_sensitive         CTCTGTTTCACATTGTTAGAAAAGGAGCGATTTCGGCATCACTAAGTTCGTTCTACAGCA
ILRAD700_sensitive      CTCTGTTTCACATTGTTAGAAAAGGAGCGATTTCGGCATCACTAAGTTCGTTCTACAGCA
                        ************************************************************

H65_resistant           AAATGGGGTTACTGGGGTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTCT
H89_resistant           AAATGGGGTTACTGGGGTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTCT
H988_sensitive          AAATGGGGTTACTGGGGTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTCT
H1008_sensitive         AAATGGGGTTACTGGGGTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTCT
ILRAD700_sensitive      AAATGGGGTTACTGGGGTTTGAGTCGCCAGCGGCGTTTGTCGTCTATTTGAGCTTCCTCT
                        ************************************************************

H65_resistant           CGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTACTGTTTGTATAAGTACTTTA
H89_resistant           CGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTACTGTTTGTATAAGTACTTTA
H988_sensitive          CGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTACTGTTTGTATAAGTACTTTA
H1008_sensitive         CGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTACTGTTTGTATAAGTACTTTA
ILRAD700_sensitive      CGTTTGGCATGTCTTTGATGCTCTCCGCCAACGCTGTGTACTGTTTGTATAAGTACTTTA
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```
H65_resistant          CACATTTCTACAAGCTTGTGCAGGGCGACCCGGAAGCTAAGCCTGAGGATGAAAGATTCT
H89_resistant          CACATTTCTACAAGCTTGTGCAGGGCGACCCGGAAGCTAAGCCTGAGGATGAAAGATTCT
H988_sensitive         CACATTTCTACAAGCTTGTGCAGGGCGACCCGGAAGCTAAGCCTGAGGATGAAAGATTCT
H1008_sensitive        CACATTTCTACAAGCTTGTGCAGGGCGACCCGGAAGCTAAGCCTGAGGATGAAAGATTCT
ILRAD700_sensitive     CACATTTCTACAAGCTTGTGCAGGGCGACCCGGAAGCTAAGCCTGAGGATGAAAGATTCT
                       ************************************************************

H65_resistant          GGACGAACATTTACACGTACTACAACGTGATCATCTTTTCAACGCAGGTTGTTGCCGAGA
H89_resistant          GGACGAACATTTACACGTACTACAACGTGATCATCTTTTCAACGCAGGTTGTTGCCGAGA
H988_sensitive         GGACGAACATTTACACGTACTACAACGTGATCATCTTTTCAACGCAGGTTGTTGCCGAGA
H1008_sensitive        GGACGAACATTTACACGTACTACAACGTGATCATCTTTTCAACGCAGGTTGTTGCCGAGA
ILRAD700_sensitive     GGACGAACATTTACACGTACTACAACGTGATCATCTTTTCAACGCAGGTTGTTGCCGAGA
                       ************************************************************

H65_resistant          TATTCATGCTGACGCCAGTTGGCAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGGCT
H89_resistant          TATTCATGCTGACGCCAGTTGGCAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGGCT
H988_sensitive         TATTCATGCTGACGCCAGTTGGCAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGGCT
H1008_sensitive        TATTCATGCTGACGCCAGTTGGCAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGGCT
ILRAD700_sensitive     TATTCATGCTGACGCCAGTTGGCAGGAGGATCCCTCTGCACCCGAGGCTGTGCGTTGGCT
                       ************************************************************

H65_resistant          TCGCCCTCCCATTTTTTCAGCTGCTTTCGTATATGATGGCCACTACGTTCCACACGACGG
H89_resistant          TCGCCCTCCCATTTTTTCAGCTGCTTTCGTATATGATGGCCACTACGTTCCACACGACGG
H988_sensitive         TCGCCCTCCCATTTTTTCAGCTGCTTTCGTATATGATGGCCACTACGTTCCACACGACGG
H1008_sensitive        TCGCCCTCCCATTTTTTCAGCTGCTTTCGTATATGATGGCCACTACGTTCCACACGACGG
ILRAD700_sensitive     TCGCCCTCCCATTTTTTCAGCTGCTTTCGTATATGATGGCCACTACGTTCCACACGACGG
                       ************************************************************

H65_resistant          AGGCCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCAT
H89_resistant          AGGCCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCAT
H988_sensitive         AGGCCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCAT
H1008_sensitive        AGGCCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCAT
ILRAD700_sensitive     AGGCCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCAT
                       ************************************************************
H65_resistant          TCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCAT
H89_resistant          TCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCAT
H988_sensitive         TCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCAT
H1008_sensitive        TCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCAT
ILRAD700_sensitive     TCTGCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCAT
                       ************************************************************
```

```
H65_resistant           ACGTCTTTGGACTCCCACTTTCTGG
H89_resistant           ACGTCTTTGGACTCCCACTTTCTGG
H988_sensitive          ACGTCTTTGGACTCCCACTTTCTGG
H1008_sensitive         ACGTCTTTGGACTCCCACTTTCTGG
ILRAD700_sensitive      ACGTCTTTGGACTCCCACTTTCTGG
                        *************************


>TvY486_0043680F2       ccggtgcaaagacgttattc
>TvY486_0043680R2       CTGCTCAACGCTCCTCTTCT

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           CCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCATTCT
H89_resistant           CCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCATTCT
H988_sensitive          CCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCATTCT
H1008_sensitive         CCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCATTCT
ILRAD700_sensitive      CCGGTGCAAAGACGTTATTCCTTGCCATGGCATTTGTGAACGGCCTGTCAAAGTCATTCT
                        ************************************************************

H65_resistant           GCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCATACG
H89_resistant           GCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCATACG
H988_sensitive          GCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCATACG
H1008_sensitive         GCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCATACG
ILRAD700_sensitive      GCGGGTCCAGCACCGTGGCTCTCGCGGGCCCGTTTCCCACCCGGTTCATTGGAGCATACG
                        ************************************************************

H65_resistant           TCTTTGGACTCCCACTTTCTGGCGTGATCACTTCCATCCTGTCCATGTCGATCCAGGGGT
H89_resistant           TCTTTGGACTCCCACTTTCTGGCGTGATCACTTCCATCCTGTCCATGTCGATCCAGGGGT
H988_sensitive          TCTTTGGACTCCCACTTTCTGGCGTGATCACTTCCATCCTGTCCATGTCGATCCAGGGGT
H1008_sensitive         TCTTTGGACTCCCACTTTCTGGCGTGATCACTTCCATCCTGTCCATGTCGATCCAGGGGT
ILRAD700_sensitive      TCTTTGGACTCCCACTTTCTGGCGTGATCACTTCCATCCTGTCCATGTCGATCCAGGGGT
                        ************************************************************
H65_resistant           CAATGAGCAACGACTTCAACAGTCTCTTGACTCAGTCGTACATATATTTCTCCACGACAT
H89_resistant           CAATGAGCAACGACTTCAACAGTCTCTTGACTCAGTCGTACATATATTTCTCCACGACAT
H988_sensitive          CAATGAGCAACGACTTCAACAGTCTCTTGACTCAGTCGTACATATATTTCTCCACGACAT
H1008_sensitive         CAATGAGCAACGACTTCAACAGTCTCTTGACTCAGTCGTACATATATTTCTCCACGACAT
ILRAD700_sensitive      CAATGAGCAACGACTTCAACAGTCTCTTGACTCAGTCGTACATATATTTCTCCACGACAT
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H65_resistant          TGGCTTTTCAAGTCATCGCATGCGTTCTGCTGTTCCTGCTCCCCAAGAATCCGTACGCCC
H89_resistant          TGGCTTTTCAAGTCATCGCATGCGTTCTGCTGTTCCTGCTCCCCAAGAATCCGTACGCCC
H988_sensitive         TGGCTTTTCAAGTCATCGCATGCGTTCTGCTGTTCCTGCTCCCCAAGAATCCGTACGCCC
H1008_sensitive        TGGCTTTTCAAGTCATCGCATGCGTTCTGCTGTTCCTGCTCCCCAAGAATCCGTACGCCC
ILRAD700_sensitive     TGGCTTTTCAAGTCATCGCATGCGTTCTGCTGTTCCTGCTCCCCAAGAATCCGTACGCCC
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H65_resistant          TGCGCTATGCGGCGGAGCTCAGGTACGCTGTGAGGAAGAACAATGCAGGTGGCGACGCGG
H89_resistant          TGCGCTATGCGGCGGAGCTCAGGTACGCTGTGAGGAAGAACAATGCAGGTGGCGACGCGG
H988_sensitive         TGCGCTATGCGGCGGAGCTCAGGTACGCTGTGAGGAAGAACAATGCAGGTGGCGACGCGG
H1008_sensitive        TGCGCTATGCGGCGGAGCTCAGGTACGCTGTGAGGAAGAACAATGCAGGTGGCGACGCGG
ILRAD700_sensitive     TGCGCTATGCGGCGGAGCTCAGGTACGCTGTGAGGAAGAACAATGCAGGTGGCGACGCGG
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H65_resistant          GAGACAAGGACGGGCTCGAACCCCTCCCAACAAGCGAGCCCGTGAATGACGGTGACCAAG
H89_resistant          GAGACAAGGACGGGCTCGAACCCCTCCCAACAAGCGAGCCCGTGAATGACGGTGACCAAG
H988_sensitive         GAGACAAGGACGGGCTCGAACCCCTCCCAACAAGCGAGCCCGTGAATGACGGTGACCAAG
H1008_sensitive        GAGACAAGGACGGGCTCGAACCCCTCCCAACAAGCGAGCCCGTGAATGACGGTGACCAAG
ILRAD700_sensitive     GAGACAAGGACGGGCTCGAACCCCTCCCAACAAGCGAGCCCGTGAATGACGGTGACCAAG
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H65_resistant          CACAGCCGGTGGTGAGGAGCGTCCTGGACACGACGGTCGACCCGGATACGATGAAGGACA
H89_resistant          CACAGCCGGTGGTGAGGAGCGTCCTGGACACGACGGTCGACCCGGATACGATGAAGGACA
H988_sensitive         CACAGCCGGTGGTGAGGAGCGTCCTGGACACGACGGTCGACCCGGATACGATGAAGGACA
H1008_sensitive        CACAGCCGGTGGTGAGGAGCGTCCTGGACACGACGGTCGACCCGGATACGATGAAGGACA
ILRAD700_sensitive     CACAGCCGGTGGTGAGGAGCGTCCTGGACACGACGGTCGACCCGGATACGATGAAGGACA
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H65_resistant          CTGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCG
H89_resistant          CTGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCG
H988_sensitive         CTGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCG
H1008_sensitive        CTGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCG
ILRAD700_sensitive     CTGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCG
                       ************************************************************
H65_resistant          TCGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCC
H89_resistant          TCGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCC
H988_sensitive         TCGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCC
H1008_sensitive        TCGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCC
ILRAD700_sensitive     TCGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCC
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H65_resistant           TCTTCT
H89_resistant           TCTTCT
H988_sensitive          TCTTCT
H1008_sensitive         TCTTCT
ILRAD700_sensitive      TCTTCT
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>TvY486_0043680F3      tgatcaggtcgaaaacacca
>TvY486_0043680R3      GTGGTGCAAAGCAAAATGAA

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           TGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGT
H89_resistant           TGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGT
H988_sensitive          TGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGT
H1008_sensitive         TGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGT
ILRAD700_sensitive      TGATCAGGTCGAAAACACCACCAACGCAGAACAGATGTTGAAGGCGGAGATCTGGGTCGT
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H65_resistant           CGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCCT
H89_resistant           CGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCCT
H988_sensitive          CGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCCT
H1008_sensitive         CGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCCT
ILRAD700_sensitive      CGTCAAGCGCATATACCCAATCCTCGCCACGTGCTTTTTAGTCTACTGCTCAACGCTCCT
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H65_resistant           CTTCTGGCCCGGCGTCTTCATTGCTGTGGACCCAAAGGGGTGGAATTTTTGGTACACCAC
H89_resistant           CTTCTGGCCCGGCGTCTTCATTGCTGTGGACCCAAAGGGGTGGAATTTTTGGTACACCAC
H988_sensitive          CTTCTGGCCCGGCGTCTTCATTGCTGTGGACCCAAAGGGGTGGAATTTTTGGTACACCAC
H1008_sensitive         CTTCTGGCCCGGCGTCTTCATTGCTGTGGACCCAAAGGGGTGGAATTTTTGGTACACCAC
ILRAD700_sensitive      CTTCTGGCCCGGCGTCTTCATTGCTGTGGACCCAAAGGGGTGGAATTTTTGGTACACCAC
                        ************************************************************
H65_resistant           GATCATGATGGCTATGTTCAACTTTGGTGACTTCTTCTCGCGCCTGCAGCTTCAGTTCAA
H89_resistant           GATCATGATGGCTATGTTCAACTTTGGTGACTTCTTCTCGCGCCTGCAGCTTCAGTTCAA
H988_sensitive          GATCATGATGGCTATGTTCAACTTTGGTGACTTCTTCTCGCGCCTGCAGCTTCAGTTCAA
H1008_sensitive         GATCATGATGGCTATGTTCAACTTTGGTGACTTCTTCTCGCGCCTGCAGCTTCAGTTCAA
ILRAD700_sensitive      GATCATGATGGCTATGTTCAACTTTGGTGACTTCTTCTCGCGCCTGCAGCTTCAGTTCAA
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H65_resistant          GAACCTCCACCCGTCCCCGCGGACGGTGATCATCGGCGCGTTCGCTCGCCTGCTGATCAT
H89_resistant          GAACCTCCACCCGTCCCCGCGGACGGTGATCATCGGCGCGTTCGCTCGCCTGCTGATCAT
H988_sensitive         GAACCTCCACCCGTCCCCGCGGACGGTGATCATCGGCGCGTTCGCTCGCCTGCTGATCAT
H1008_sensitive        GAACCTCCACCCGTCCCCGCGGACGGTGATCATCGGCGCGTTCGCTCGCCTGCTGATCAT
ILRAD700_sensitive     GAACCTCCACCCGTCCCCGCGGACGGTGATCATCGGCGCGTTCGCTCGCCTGCTGATCAT
                       ************************************************************

H65_resistant          CGTGCCGCTCTTCCTCTGTCAGAAGAAGGTGATAGAGGGCAACTCGGCCAAGGTGCTGTG
H89_resistant          CGTGCCGCTCTTCCTCTGTCAGAAGAAGGTGATAGAGGGCAACTCGGCCAAGGTGCTGTG
H988_sensitive         CGTGCCGCTCTTCCTCTGTCAGAAGAAGGTGATAGAGGGCAACTCGGCCAAGGTGCTGTG
H1008_sensitive        CGTGCCGCTCTTCCTCTGTCAGAAGAAGGTGATAGAGGGCAACTCGGCCAAGGTGCTGTG
ILRAD700_sensitive     CGTGCCGCTCTTCCTCTGTCAGAAGAAGGTGATAGAGGGCAACTCGGCCAAGGTGCTGTG
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H65_resistant          CTTGTTCCTGTCCCTCTTCTGGGGCCTCTCGAACGGTGTCTGTGGGGGTATGATGATCAT
H89_resistant          CTTGTTCCTGTCCCTCTTCTGGGGCCTCTCGAACGGTGTCTGTGGGGGTATGATGATCAT
H988_sensitive         CTTGTTCCTGTCCCTCTTCTGGGGCCTCTCGAACGGTGTCTGTGGGGGTATGATGATCAT
H1008_sensitive        CTTGTTCCTGTCCCTCTTCTGGGGCCTCTCGAACGGTGTCTGTGGGGGTATGATGATCAT
ILRAD700_sensitive     CTTGTTCCTGTCCCTCTTCTGGGGCCTCTCGAACGGTGTCTGTGGGGGTATGATGATCAT
                       ************************************************************

H65_resistant          TTACGGGCCGAGAACTGCATCCCTGACAACAGCCGGGCAGCGCTCGATCGCCGGCATATG
H89_resistant          TTACGGGCCGAGAACTGCATCCCTGACAACAGCCGGGCAGCGCTCGATCGCCGGCATATG
H988_sensitive         TTACGGGCCGAGAACTGCATCCCTGACAACAGCCGGGCAGCGCTCGATCGCCGGCATATG
H1008_sensitive        TTACGGGCCGAGAACTGCATCCCTGACAACAGCCGGGCAGCGCTCGATCGCCGGCATATG
ILRAD700_sensitive     TTACGGGCCGAGAACTGCATCCCTGACAACAGCCGGGCAGCGCTCGATCGCCGGCATATG
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H65_resistant          CAACAACGTGTCACTGCTAATGGGCCTCTTCTTGGGGTCGGCGGGGGCACTGGGTTTGGA
H89_resistant          CAACAACGTGTCACTGCTAATGGGCCTCTTCTTGGGGTCGGCGGGGGCACTGGGTTTGGA
H988_sensitive         CAACAACGTGTCACTGCTAATGGGCCTCTTCTTGGGGTCGGCGGGGGCACTGGGTTTGGA
H1008_sensitive        CAACAACGTGTCACTGCTAATGGGCCTCTTCTTGGGGTCGGCGGGGGCACTGGGTTTGGA
ILRAD700_sensitive     CAACAACGTGTCACTGCTAATGGGCCTCTTCTTGGGGTCGGCGGGGGCACTGGGTTTGGA
                       ************************************************************
H65_resistant          GAAGCACTTGTGATATCACTAGGATCTAAGGTAGATGCTAATCTTCATTGAGTAAAGCGT
H89_resistant          GAAGCACTTGTGATATCACTAGGATCTAAGGTAGATGCTAATCTTCATTGAGTAAAGCGT
H988_sensitive         GAAGCACTTGTGATATCACTAGGATCTAAGGTAGATGCTAATCTTCATTGAGTAAAGCGT
H1008_sensitive        GAAGCACTTGTGATATCACTAGGATCTAAGGTAGATGCTAATCTTCATTGAGTAAAGCGT
ILRAD700_sensitive     GAAGCACTTGTGATATCACTAGGATCTAAGGTAGATGCTAATCTTCATTGAGTAAAGCGT
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H65_resistant           TAAGAGGTCTAAACAGAAAAAACGGGCAAAAAACTACACCTCCCCACTTTCCCCCTCTGT
H89_resistant           TAAGAGGTCTAAACAGAAAAAACGGGCAAAAAACTACACCTCCCCACTTTCCCCCTCTGT
H988_sensitive          TAAGAGGTCTAAACAGAAAAAACGGGCAAAAAACTACACCTCCCCACTTTCCCCCTCTGT
H1008_sensitive         TAAGAGGTCTAAACAGAAAAAACGGGCAAAAAACTACACCTCCCCACTTTCCCCCTCTGT
ILRAD700_sensitive      TAAGAGGTCTAAACAGAAAAAACGGGCAAAAAACTACACCTCCCCACTTTCCCCCTCTGT
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H65_resistant           TTTCTCCACATATGTAAACGCATGTGGTGCAAAGCAAAATGAA
H89_resistant           TTTCTCCACATATGTAAACGCATGTGGTGCAAAGCAAAATGAA
H988_sensitive          TTTCTCCACATATGTAAACGCATGTGGTGCAAAGCAAAATGAA
H1008_sensitive         TTTCTCCACATATGTAAACGCATGTGGTGCAAAGCAAAATGAA
ILRAD700_sensitive      TTTCTCCACATATGTAAACGCATGTGGTGCAAAGCAAAATGAA
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Sequencing performed on TvY486_0202110 using 2 resistant and 3 sensitive *T. vivax* strains

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>TvY486_0202110F1       GCAAACATGATTCTTTCTCTTTGAT
>TvY486_0202110R1       ataatataccggtgatgactccaga

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H65_resistant           GCAAACATGATTCTTTCTCTTTGATCTAGTCAAGGGACACGCACCCTCCACTGCCAAATA
H988_sensitive          GCAAACATGATTCTTTCTCTTTGATCTAGTCAAGGGACACGCACCCTCCACTGCCAAATA
H1008_sensitive         GCAAACATGATTCTTTCTCTTTGATCTAGTCAAGGGACACGCACCCTCCACTGCCAAATA
ILRAD700_sensitive      GCAAACATGATTCTTTCTCTTTGATCTAGTCAAGGGACACGCACCCTCCACTGCCAAATA
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H65_resistant           GATTTTCCAGAAAATGGCAGTGTTAGGGTTTGAGTCCACCTCGGCCCTGCTTGTTTACGT
H988_sensitive          GATTTTCCAGAAAATGGCAGTGTTAGGGTTTGAGTCCACCTCGGCCCTGCTTGTTTACGT
H1008_sensitive         GATTTTCCAGAAAATGGCAGTGTTAGGGTTTGAGTCCACCTCGGCCCTGCTTGTTTACGT
ILRAD700_sensitive      GATTTTCCAGAAAATGGCAGTGTTAGGGTTTGAGTCCACCTCGGCCCTGCTTGTTTACGT
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H65_resistant           GAGCTTCCTGTTCTTTGGGATGTCGCTGATGCTGACCGCCAACTCTATATATTCCTTATA
H988_sensitive          GAGCTTCCTGTTCTTTGGGATGTCGCTGATGCTGACCGCCAACTCTATATATTCCTTATA
H1008_sensitive         GAGCTTCCTGTTCTTTGGGATGTCGCTGATGCTGACCGCCAACTCTATATATTCCTTATA
ILRAD700_sensitive      GAGCTTCCTGTTCTTTGGGATGTCGCTGATGCTGACCGCCAACTCTATATATTCCTTATA
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H65_resistant           CGGCTACTCCACAGAGTTTTACAGATTGGCACAGGGCGACCCGAATGCCACAACCAGCGA
H988_sensitive          CGGCTACTCCACAGAGTTTTACAGATTGGCACAGGGCGACCCGAATGCCACAACCAGCGA
H1008_sensitive         CGGCTACTCCACAGAGTTTTACAGATTGGCACAGGGCGACCCGAATGCCACAACCAGCGA
ILRAD700_sensitive      CGGCTACTCCACAGAGTTTTACAGATTGGCACAGGGCGACCCGAATGCCACAACCAGCGA
                        ************************************************************

H65_resistant           CCCCGACTTCTGGAAGAATATCTACACATATTATAACGTGGTGTTGTTTAGCCTGCAACT
H988_sensitive          CCCCGACTTCTGGAAGAATATCTACACATATTATAACGTGGTGTTGTTTAGCCTGCAACT
H1008_sensitive         CCCCGACTTCTGGAAGAATATCTACACATATTATAACGTGGTGTTGTTTAGCCTGCAACT
ILRAD700_sensitive      CCCCGACTTCTGGAAGAATATCTACACATATTATAACGTGGTGTTGTTTAGCCTGCAACT
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H65_resistant           TTCTACAGAGCTATTCATGCTGACTCCGCTCGGAAGAAGGATCCCATTGCGGCTGAGGTT
H988_sensitive          TTCTACAGAGCTATTCATGCTGACTCCGCTCGGAAGAAGGATCCCATTGCGGCTGAGGTT
H1008_sensitive         TTCTACAGAGCTATTCATGCTGACTCCGCTCGGAAGAAGGATCCCATTGCGGCTGAGGTT
ILRAD700_sensitive      TTCTACAGAGCTATTCATGCTGACTCCGCTCGGAAGAAGGATCCCATTGCGGCTGAGGTT
                        ************************************************************

H65_resistant           GGCGCTAGGCTTCTCCCTTTCGTTCGTGCAGCTCCTTTCGTACATGATGGTCACCACATT
H988_sensitive          GGCGCTAGGCTTCTCCCTTTCGTTCGTGCAGCTCCTTTCGTACATGATGGTCACCACATT
H1008_sensitive         GGCGCTAGGCTTCTCCCTTTCGTTCGTGCAGCTCCTTTCGTACATGATGGTCACCACATT
ILRAD700_sensitive      GGCGCTAGGCTTCTCCCTTTCGTTCGTGCAGCTCCTTTCGTACATGATGGTCACCACATT
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H65_resistant           CCATACAAGCGAATCCGGCGCAAAGTGTGTATTTTTGTTCAGTGCTTTTGTCAATGGAAT
H988_sensitive          CCATACAAGCGAATCCGGCGCAAAGTGTGTATTTTTGTTCAGTGCTTTTGTCAATGGAAT
H1008_sensitive         CCATACAAGCGAATCCGGCGCAAAGTGTGTATTTTTGTTCAGTGCTTTTGTCAATGGAAT
ILRAD700_sensitive      CCATACAAGCGAATCCGGCGCAAAGTGTGTATTTTTGTTCAGTGCTTTTGTCAATGGAAT
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H65_resistant           TGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCCAACAAAATT
H988_sensitive          TGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCCAACAAAATT
H1008_sensitive         TGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCCAACAAAATT
ILRAD700_sensitive      TGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCCAACAAAATT
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H65_resistant           TTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTATATTAT
H988_sensitive          TTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTATATTAT
H1008_sensitive         TTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTATATTAT
ILRAD700_sensitive      TTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTATATTAT
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>TvY486_0202110F2       caatggaattgaaaaatctctctgt
>TvY486_0202110R2       ggaaaaacaaaactgttgtgaaata

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           CAATGGAATTGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCC
H988_resistant          CAATGGAATTGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCC
H1008_sensitive         CAATGGAATTGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCC
ILRAD700_sensitive      CAATGGAATTGAAAAATCTCTCTGTGGCTCCAGCACTGTGGCACTTGCAGGTCCCTTTCC
                        ************************************************************

H65_resistant           AACAAAATTTTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTAT
H988_resistant          AACAAAATTTTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTAT
H1008_sensitive         AACAAAATTTTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTAT
ILRAD700_sensitive      AACAAAATTTTTTGCGGCTGTCATCCTTGGTATCCCATTTTCTGGAGTCATCACCGGTAT
                        ************************************************************

H65_resistant           ATTATCCGTGACGGTTAAGGCATCGATGGATGGGGACTTTCACAGTTTGCTCCATCAGTC
H988_resistant          ATTATCCGTGACGGTTAAGGCATCGATGGATGGGGACTTTCACAGTTTGCTCCATCAGTC
H1008_sensitive         ATTATCCGTGACGGTTAAGGCATCGATGGATGGGGACTTTCACAGTTTGCTCCATCAGTC
ILRAD700_sensitive      ATTATCCGTGACGGTTAAGGCATCGATGGATGGGGACTTTCACAGTTTGCTCCATCAGTC
                        ************************************************************

H65_resistant           ATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCCTTTTGTATTT
H988_resistant          ATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCCTTTTGTATTT
H1008_sensitive         ATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCCTTTTGTATTT
ILRAD700_sensitive      ATACATATATTTTTCCATCGCTATGGTTTTTCAGTCGGTGACCTGCGTCCTTTTGTATTT
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H65_resistant           GCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACGCGGCGAGGGG
H988_resistant          GCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACGCGGCGAGGGG
H1008_sensitive         GCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACGCGGCGAGGGG
ILRAD700_sensitive      GCTTCCGCGAAATCCGTATGCTTTACGCTATGCTGCAGAATTTAGGTACGCGGCGAGGGG
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H65_resistant           TAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTGCACCTGATTC
H988_resistant          TAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTGCACCTGATTC
H1008_sensitive         TAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTGCACCTGATTC
ILRAD700_sensitive      TAATCCTGTTGAGTGTGAGGAGCAGACGGAAAAGAAGGAAGCAAATGGTGCACCTGATTC
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H65_resistant           GCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGTACAACACTGC
H988_resistant          GCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGTACAACACTGC
H1008_sensitive         GCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGTACAACACTGC
ILRAD700_sensitive      GCGTCCAGCGAAAGGACCGGCAGATGACTATTGTGACGATGCGCAGCCGTACAACACTGC
                        ************************************************************

H65_resistant           AAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTGACCAGGTGGA
H988_resistant          AAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTGACCAGGTGGA
H1008_sensitive         AAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTGACCAGGTGGA
ILRAD700_sensitive      AAAAAACGTCTTGGATACATCGATTGATCCGGACACGATGAAGGATACTGACCAGGTGGA
                        ************************************************************

H65_resistant           GAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCATCAAGCGCAT
H988_resistant          GAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCATCAAGCGCAT
H1008_sensitive         GAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCATCAAGCGCAT
ILRAD700_sensitive      GAACACCACTAGTGCTGAGCAGATGCTGAAGGCAGAAGTGTGGGTTGTCATCAAGCGCAT
                        ************************************************************

H65_resistant           ATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGTTTTTCC
H988_resistant          ATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGTTTTTCC
H1008_sensitive         ATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGTTTTTCC
ILRAD700_sensitive      ATACCCGGTCCTTTCTACGTGCTTTTTTGTCTATTTCACAACAGTTTTGTTTTTCC
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>TvY486_0202110F3       ctatttcacaacagttttgtttttcc
>TvY486_0202110R3       acaaacacttatttaccacacaggtt


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H65_resistant           CTATTTCACAACAGTTTTGTTTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTG
H89_resistant           CTATTTCACAACAGTTTTGTTTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTG
H988_sensitive          CTATTTCACAACAGTTTTGTTTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTG
H1008_sensitive         CTATTTCACAACAGTTTTGTTTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTG
ILRAD700_sensitive      CTATTTCACAACAGTTTTGTTTTTCCCTGGTGTTTTTATCTCGGTGGATTACAAGGGCTG
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H65_resistant          GAATCACTGGTACGGCACTGCGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTG
H89_resistant          GAATCACTGGTACGGCACTGCGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTG
H988_sensitive         GAATCACTGGTACGGCACTGCGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTG
H1008_sensitive        GAATCACTGGTACGGCACTGCGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTG
ILRAD700_sensitive     GAATCACTGGTACGGCACTGCGGTGATGGTTGTGTTCAATTTGGGTGATTTTGTTTCGTG
                       ************************************************************

H65_resistant          TATGTTTCTTCAGTTCAAGAGAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATT
H89_resistant          TATGTTTCTTCAGTTCAAGAGAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATT
H988_sensitive         TATGTTTCTTCAGTTCAAGAGAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATT
H1008_sensitive        TATGTTTCTTCAGTTCAAGAGAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATT
ILRAD700_sensitive     TATGTTTCTTCAGTTCAAGAGAAATCACCCATCACCGAAGGCAGTGATTATAGGGTCATT
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H65_resistant          CGCTCGCCTACTGATTGCAGTACCGCTTTTCCTTTGCCAACGTCGTATAATAGAGGGCCA
H89_resistant          CGCTCGCCTACTGATTGCAGTACCGCTTTTCCTTTGCCAACGTCGTATAATAGAGGGCCA
H988_sensitive         CGCTCGCCTACTGATTGCAGTACCGCTTTTCCTTTGCCAACGTCGTATAATAGAGGGCCA
H1008_sensitive        CGCTCGCCTACTGATTGCAGTACCGCTTTTCCTTTGCCAACGTCGTATAATAGAGGGCCA
ILRAD700_sensitive     CGCTCGCCTACTGATTGCAGTACCGCTTTTCCTTTGCCAACGTCGTATAATAGAGGGCCA
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H65_resistant          TGCCGCCAAAGCGTTGTCCTGTGTTCTTTCACTCCTCTGGGGTATGACCAACGGTTTTTG
H89_resistant          TGCCGCCAAAGCGTTGTCCTGTGTTCTTTCACTCCTCTGGGGTATGACCAACGGTTTTTG
H988_sensitive         TGCCGCCAAAGCGTTGTCCTGTGTTCTTTCACTCCTCTGGGGTATGACCAACGGTTTTTG
H1008_sensitive        TGCCGCCAAAGCGTTGTCCTGTGTTCTTTCACTCCTCTGGGGTATGACCAACGGTTTTTG
ILRAD700_sensitive     TGCCGCCAAAGCGTTGTCCTGTGTTCTTTCACTCCTCTGGGGTATGACCAACGGTTTTTG
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H65_resistant          TGGCGGTATGATGCTTATTTACGGACCAAGGACCGCGTCGCTTACAACGGCAGGCCAGCG
H89_resistant          TGGCGGTATGATGCTTATTTACGGACCAAGGACCGCGTCGCTTACAACGGCAGGCCAGCG
H988_sensitive         TGGCGGTATGATGCTTATTTACGGACCAAGGACCGCGTCGCTTACAACGGCAGGCCAGCG
H1008_sensitive        TGGCGGTATGATGCTTATTTACGGACCAAGGACCGCGTCGCTTACAACGGCAGGCCAGCG
ILRAD700_sensitive     TGGCGGTATGATGCTTATTTACGGACCAAGGACCGCGTCGCTTACAACGGCAGGCCAGCG
                       ************************************************************

H65_resistant          TTCTCTTGCCGGGATATGCAACAATGTGTCACTACTGGTGGGCCTTTTTGCAGGTTCGGC
H89_resistant          TTCTCTTGCCGGGATATGCAACAATGTGTCACTACTGGTGGGCCTTTTTGCAGGTTCGGC
H988_sensitive         TTCTCTTGCCGGGATATGCAACAATGTGTCACTACTGGTGGGCCTTTTTGCAGGTTCGGC
H1008_sensitive        TTCTCTTGCCGGGATATGCAACAATGTGTCACTACTGGTGGGCCTTTTTGCAGGTTCGGC
ILRAD700_sensitive     TTCTCTTGCCGGGATATGCAACAATGTGTCACTACTGGTGGGCCTTTTTGCAGGTTCGGC
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H65_resistant           AGCGGCAATAGGACTGAGCAAGACGCTGTAATGTGAGTGGGTTCCAGAAAAGGTTTAAGA
H89_resistant           AGCGGCAATAGGACTGAGCAAGACGCTGTAATGTGAGTGGGTTCCAGAAAAGGTTTAAGA
H988_sensitive          AGCGGCAATAGGACTGAGCAAGACGCTGTAACGTGAGTGGGTTCCAGAAAAGGTTTAAGA
H1008_sensitive         AGCGGCAATAGGACTGAGCAAGACGCTGTAACGTGAGTGGGTTCCAGAAAAGGTTTAAGA
ILRAD700_sensitive      AGCGGCAATAGGACTGAGCAAGACGCTGTAACGTGAGTGGGTTCCAGAAAAGGTTTAAGA
                        ****************************** ****************************

H65_resistant           GTATACTTATTGTTATTTTTTTCTATTTTTAACCTGTGTGGTAAATAAGTGTTTAA
H89_resistant           GTATACTTATTGTTATTTTTTTCTATTTTTAACCTGTGTGGTAAATAAGTGTTTGT
H988_sensitive          GTATACTTATTGTTATTTTTTTCTATTTTTAACCTGTGTGGTAAATAAGTGTTTGT
H1008_sensitive         GTATACTTATTGTTATTTTTTTCTATTTTTAACCTGTGTGGTAAATAAGTGTTTGT
ILRAD700_sensitive      GTATACTTATTGTTATTTTTTTCTATTTTTAACCTGTGTGGTAAATAAGTGTTTGT
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Sequencing performed on TvY486_1103740 using 2 resistant and 3 sensitive *T. vivax* strains

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>TvY486_1103740F1       TGTTGTCCAGCTGCTTTCCTA
>TvY486_1103740R1       TTCTCCATCGACCCCTTGAT


CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           TGTTGTCCAGCTGCTTTCCTAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGC
H89_resistant           TGTTGTCCAGCTGCTTTCCTAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGC
H988_sensitive          TGTTGTCCAGCTGCTTTCCTAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGC
H1008_sensitive         TGTTGTCCAGCTGCTTTCCTAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGC
ILRAD700_sensitive      TGTTGTCCAGCTGCTTTCCTAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGC
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H65_resistant           TTCGGAGGTCTACGTGTATGTGACCTGCGTTATCCTTGGCATTGCGATTCTCACGCCCCT
H89_resistant           TTCGGAGGTCTACGTGTATGTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCT
H988_sensitive          TTCGGAGGTCTACGTGTATGTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCT
H1008_sensitive         TTCGGAGGTCTACGTGTATGTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCT
ILRAD700_sensitive      TTCGGAGGTCTACGTGTATGTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCT
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H65_resistant           CAAGTGCCTGGTGTCTGCACCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGA
H89_resistant           CAAGTGCCTGGTGTCTGCACCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGA
H988_sensitive          CAAGTGCCTGGTGTCTGCACCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGA
H1008_sensitive         CAAGTGCCTGGTGTCTGCACCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGA
ILRAD700_sensitive      CAAGTGCCTGGTGTCTGCACCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGA
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H65_resistant           TCCGGACGCCAAGCCAACCCCCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGTGC
H89_resistant           TCCGGACGCCAAGCCAACTCCCCCCATCTTCTGGGCTAACATTCTCACATTCTAAAGTGC
H988_sensitive          TCCGGACGCCAAGCCAACCCCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGCGC
H1008_sensitive         TCCGGACGCCAAGCCAACCCCCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGTGC
ILRAD700_sensitive      TCCGGACGCCAAGCCAACCCCCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGTGC
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H65_resistant          AGTGTCATTGGTGACGCAGATACTTTTTGGACCCACAGTTCTCACTCGCACGGTCAGGCG
H89_resistant          AGTGTCATTGGTGACACAAATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCG
H988_sensitive         AGTGTCATTGGTGACACAAATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCG
H1008_sensitive        AGTGTCATTGGTGACGCAGATACTTTTTGGACCCACAGTTCTCACTCGCACGGTCAGGCG
ILRAD700_sensitive     AGTGTCATTGGTGACGCAAATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCG
                       *************** ** ***** ** *  ****************************

H65_resistant          CCTCTCCTTGAGTACGCGCTTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGC
H89_resistant          CCTCTCCTTGAGTACGCGCTTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGC
H988_sensitive         CCTCTCCTTGAGTACGCGCTTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGC
H1008_sensitive        CCTCTCCTTGAGTACGCGCTTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGC
ILRAD700_sensitive     CCTCTCCTTGAGTACGCGCTTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGC
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H65_resistant          TGTTCTCTTCATGCCTGTAGTGAAAGTTCCGCAGACTGTCGCCATAGTTGTGTTTTTCAT
H89_resistant          TGTTCTCTTCATGCCTGTAGTGAAAGTTATGCAGACTGTCGCCATAGTTGTGTTTTTCAT
H988_sensitive         TGTTCTCTTCATGCCTGTAGTGAAAGTTACGCAGACTGTCGCCATAGTTGTGTTTTTCAT
H1008_sensitive        TGTTCTCTTCATGCCTGTAGTGAAAGTTCCGCAGACTGTCGCCATAGTTGTGTTTTTCAT
ILRAD700_sensitive     TGTTCTCTTCATGCCTGTAGTGAAAGTTACGCAGACTGTCGCCATAGTTGTGTTTTTCAT
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H65_resistant          TGCCATTATACTGTCTGGGATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGT
H89_resistant          TGTCATTATACTGTCTGGGATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGT
H988_sensitive         TGTCATTATACTGTCTGGGATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGT
H1008_sensitive        TGCCATTATACTGTCTGGGATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGT
ILRAD700_sensitive     TGCCATTATACTGTCTGGGATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGT
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H65_resistant          GTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTGCTCGTTCTCTGGCGT
H89_resistant          GTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTGCTCGTTCTCTGGCGT
H988_sensitive         GTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTGCTCGTTCTCTGGCGT
H1008_sensitive        GTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTGCTCGTTCTCTGGCGT
ILRAD700_sensitive     GTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTGCTCGTTCTCTGGCGT
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H65_resistant          GATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H89_resistant          GATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H988_sensitive         GATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H1008_sensitive        GATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
ILRAD700_sensitive     GATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
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>TvY486_1103740F2       ACATATGGAGGCCACCACTTAC
>TvY486_1103740R2       ATCACCAAAGTTGTAGCACAGAATC
CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           ACATATGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGG
H89_resistant           ACATATGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGG
H988_sensitive          ACATATGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGG
H1008_sensitive         ACATATGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGG
ILRAD700_sensitive      ACATATGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGG
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H65_resistant           TGCCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAA
H89_resistant           TGCCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAA
H988_sensitive          TGCCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAA
H1008_sensitive         TGCCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAA
ILRAD700_sensitive      TGCCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAA
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H65_resistant           GGGGTTGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCT
H89_resistant           GGGGTTGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCT
H988_sensitive          GGGGTTGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCT
H1008_sensitive         GGGGTTGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCT
ILRAD700_sensitive      GGGGTTGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCT
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H65_resistant           TGGGATGGTGATTATAGCCGCCTCACTTGTCATGACTTATTCGCTGCGCTGCAATTCCTA
H89_resistant           TGGGATGGTGATTATAGCCGCCTCACTTGTCATGACTTATTCGCTGCGCTGCAATTCCTA
H988_sensitive          TGGGATGGTGATTATAGCCGCCTCACTTGTCATGACTTATTCGCTGCGCTGCAATTCCTA
H1008_sensitive         TGGGATGGTGATTATAGCCGCCTCACTTGTCATGACTTATTCGCTGCGCTGCAATTCCTA
ILRAD700_sensitive      TGGGATGGTGATTATAGCCGCCTCACTTGTCATGACTTATTCGCTGCGCTGCAATTCCTA
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H65_resistant           CGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGG
H89_resistant           CGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGG
H988_sensitive          CGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGG
H1008_sensitive         CGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGG
ILRAD700_sensitive      CGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGG
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H65_resistant          ATGCCACAACGGCACAGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGAGAATGA
H89_resistant          ATGCCACAACGGCACAGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGAGAATGA
H988_sensitive         ATGCCACAACGGCACAGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGAGAATGA
H1008_sensitive        ATGCCACAACGGCACAGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGAGAATGA
ILRAD700_sensitive     ATGCCACAACGGCACAGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGAGAATGA
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H65_resistant          TGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCC
H89_resistant          TGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCC
H988_sensitive         TGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCC
H1008_sensitive        TGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCC
ILRAD700_sensitive     TGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCC
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H65_resistant          TGTATTAAAGAAAATACACCTGATGATGACCACGTGCTCCATTTCCTTCTTTGTCACGCT
H89_resistant          TGTATTAAAGAAAATACACCTGATGATGACCACGTGCTCCATTTCCTTCTTTGTCACGCT
H988_sensitive         TGTATTAAAGAAAATACACCTGATGATGACCACGTGCTCCATTTCCTTCTTTGTCACGCT
H1008_sensitive        TGTATTAAAGAAAATACACCTGATGATGACCACGTGCTCCATTTCCTTCTTTGTCACGCT
ILRAD700_sensitive     TGTATTAAAGAAAATACACCTGATGATGACCACGTGCTCCATTTCCTTCTTTGTCACGCT
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H65_resistant          CTTTGTATTTCCAACGCTTATCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCT
H89_resistant          CTTTGTATTTCCAACGCTTATCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCT
H988_sensitive         CTTTGTATTTCCAACGCTTATCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCT
H1008_sensitive        CTTTGTATTTCCAACGCTTATCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCT
ILRAD700_sensitive     CTTTGTATTTCCAACGCTTATCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCT
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H65_resistant          TGCGATTCTGTGCTACAACTTTGGTGAT
H89_resistant          TGCGATTCTGTGCTACAACTTTGGTGAT
H988_sensitive         TGCGATTCTGTGCTACAACTTTGGTGAT
H1008_sensitive        TGCGATTCTGTGCTACAACTTTGGTGAT
ILRAD700_sensitive     TGCGATTCTGTGCTACAACTTTGGTGAT
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>TvY486_1103740F3       TTCCTTCTTTGTCACGCTCTTTAT
>TvY486_1103740R3       CAGTAAACGCTGCTACGTCTTAAA

CLUSTAL 2.0.12 multiple sequence alignment

H65_resistant           TTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCA
H89_resistant           TTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCA
H988_sensitive          TTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCA
H1008_sensitive         TTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCA
ILRAD700_sensitive      TTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCA
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H65_resistant           CAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTT
H89_resistant           CAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTT
H988_sensitive          CAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTT
H1008_sensitive         CAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTT
ILRAD700_sensitive      CAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTT
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H65_resistant           TGGCACAACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTC
H89_resistant           TGGCACAACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTC
H988_sensitive          TGGCACAACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTC
H1008_sensitive         TGGCACAACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTC
ILRAD700_sensitive      TGGCACAACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTC
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H65_resistant           CCGCTTTCTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGC
H89_resistant           CCGCTTTCTCTTCACTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGC
H988_sensitive          CCGCTTTCTCTTCACTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATAC
H1008_sensitive         CCGCTTTCTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGC
ILRAD700_sensitive      CCGCTTTCTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGC
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H65_resistant           AGTACCTTACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGAT
H89_resistant           AGTACCTTACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGAT
H988_sensitive          AGTACCTTACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGAT
H1008_sensitive         AGTACCTTACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGAT
ILRAD700_sensitive      AGTACCTTACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGAT
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H65_resistant           GGTGTACGGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCA
H89_resistant           GGTGTACGGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCA
H988_sensitive          GGTGTACGGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCA
H1008_sensitive         GGTGTACGGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCA
ILRAD700_sensitive      GGTGTACGGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCA
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H65_resistant           GCTGATGGGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGT
H89_resistant           GCTGATGGGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGT
H988_sensitive          GCTGATGGGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGT
H1008_sensitive         GCTGATGGGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGT
ILRAD700_sensitive      GCTGATGGGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGT
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H65_resistant           CGTGTACGCTTTGCCCTAAGGCCACAAGTTACAACATTCAGCAACATAATATTTTTCTTT
H89_resistant           CGTGTACGCTTTGCCCTAAGGCCACAAGTTACAACATTCAGCAACATAATATTTTTCTTT
H988_sensitive          CGTGTACGCTTTGCCCTAAGGCCACAAGTTACAACATTCAGCAACATAATATTTTTCTTT
H1008_sensitive         CGTGTACGCTTTGCCCTAAGGCCACAAGTTACAACATTCAGCAACATAATATTTTTCTTT
ILRAD700_sensitive      CGTGTACGCTTTGCCCTAAGGCCACAAGTTACAACATTCAGCAACATAATATTTTTCTTT
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H65_resistant           AAGACGTAGCAGCGTTTACTG
H89_resistant           AAGACGTAGCAGCGTTTACTG
H988_sensitive          AAGACGTAGCAGCGTTTACTG
H1008_sensitive         AAGACGTAGCAGCGTTTACTG
ILRAD700_sensitive      AAGACGTAGCAGCGTTTACTG
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Sequencing performed on TvY486_0041960 using 2 resistant and 3 sensitive *T. vivax* strains

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TvY486_0041960F1        TGCTGAAGTGACATCGAAGG
TvY486_0041960R1        ACGACACCAGCGTGTAAGTG


CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           TGCTGAAGTGACATCGAAGGTTATAAAAGGTTCTTTCCTGCCCCCACCGCACTCGAGGCA
H89_resistant           TGCTGAAGTGACATCGAAGGTTATAAAAGGTTCTTTCCTGCCCCCACCGCACTCGAGGCA
H988_sensitive          TGCTGAAGTGACATCGAAGGTTATAAAAGGTTCTTTCCTGCCCCCACCGCACTCGAGGCA
H1008_sensitive         TGCTGAAGTGACATCGAAGGTTATAAAAGGTTCTTTCCTGCCCCCACCGCACTCGAGGCA
ILRAD700_sensitive      TGCTGAAGTGACATCGAAGGTTATAAAAGGTTCTTTCCTGCCCCCACCGCACTCGAGGCA
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H65_resistant           CCGTACGCAACGCACCTGACGCGCTCTGTTCTCCGCCTGCCTGTTGTCCGGCTGCTTTCC
H89_resistant           CCGTACGCAACGCACCTGACGCGCTCTGTTCTCCGCCTGCCTGTTGTCCAGCTGCTTTCC
H988_sensitive          CCGTACGCAACGCACCTGACGCGCTCTGTTCTCCGCCTGCCTGTTGTCCAGCTGCTTTCT
H1008_sensitive         CCGTACGCAACGCACCTGACGCGCTCTGTTCTCCGCCTGCCTGTTGTCCAGCTGCTTTCC
ILRAD700_sensitive      CCGTACGCAACGCACCTGACGCGCTCTGTTCTCCGCCTGCCTGTTGTCCAGCTGCTTTCT
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H65_resistant           TAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGCTTCGGAGGTCTACGTGTAT
H89_resistant           TAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGCTTCGGAGGTCTACGTGTAT
H988_sensitive          TAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGCTTCGGAGGTCTACGTGTAT
H1008_sensitive         TAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGCTTCGGAGGTCTACGTGTAT
ILRAD700_sensitive      TAGCTGCCGGCGCGCACAATGCTTTTTGGATTTACCTCCGCTTCGGAGGTCTACGTGTAT
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H65_resistant           GTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCTCAAGTGCCTGGTGTCTGCA
H89_resistant           GTGACCTGCATTATCCTTGGCATTGCGATTCTCACGCCCCTCAAGTGCCTGGTGTCTGCA
H988_sensitive          GTGACCTGCGTTATCCTTGGCATTGCGATTCTCACGCCCCTCAAGTGCCTGGTGTCTGCA
H1008_sensitive         GTGACCTGCGTTATCCTTGGCATTGCGATTCTCACGCCCCTCAAGTGCCTGGTGTCTGCA
ILRAD700_sensitive      GTGACCTGCGTTATCCTTGGCATTGCGATTCTCACGCCCCTCAAGTGCCTGGTGTCTGCA
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H65_resistant          CCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGATCCGGACGCCAAGCCAACC
H89_resistant          CCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGATCCGGACGCCAAGCCAACC
H988_sensitive         CCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGATCCGGACGCCAAGCCAACC
H1008_sensitive        CCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGATCCGGACGCCAAGCCAACC
ILRAD700_sensitive     CCGCGCTTTATGGTCGACTACTACAAGTATGTGTCGGGTGATCCGGACGCCAAGCCAACC
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H65_resistant          CCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGTGCAGTGTCATTGGTGACACAA
H89_resistant          CCCCCCATCTTCTGGGCTAACATTCTCACATTCTAAAGTGCAGTGTCATTGGTGACACAA
H988_sensitive         CCCCCCATCTCCTGGGCTAACATTCTCACATTCTACAGTGCAGTGTCATTGGTGACGCAG
H1008_sensitive        CCCCCCATCTTCTGGGCTAACATTCTCACATTCTACAGTGCAGTGTCATTGGTGACGCAA
ILRAD700_sensitive     CCCCCCATCTCCTGGGCTAACATTCTCACATTCTACAGTGCAGTGTCATTGGTGACGCAG
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H65_resistant          ATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGC
H89_resistant          ATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGC
H988_sensitive         ATACTTTTTGGACCCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGC
H1008_sensitive        ATACTCTTCGCCCCCACAGTTCTCACTCGCACGGTCAGGCGCCGCTCCTTGAGTACGCGC
ILRAD700_sensitive     ATACTTTTTGGACCCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGC
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H65_resistant          TTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGCTGTTCTCTTCATGCCTGTA
H89_resistant          TTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGCTGTTCTCTTCATGCCTGTA
H988_sensitive         TTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGCTGTTCTCTTCATGCCTGTA
H1008_sensitive        TTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGCTGTTCTCTTCATGCCTGTA
ILRAD700_sensitive     TTCACCCTTGCCATCACATCCATGATGATTGAGATCGTGGCTGTTCTCTTCATGCCTGTA
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H65_resistant          GTGAAAGTTACGCAGACTGTCGCCATAGTTGTGTTTTTCATTGCCATTATACTGTCTGGG
H89_resistant          GTGAAAGTTACGCAGACTGTCGCCATAGTTGTGTTTTTCATTGTCATTATACTGTCTGGG
H988_sensitive         GTGAAAGTTCCGCAGACTGTCGCCATAGTTGTGTTTTTCATTGCCATTATACTGTCTGGG
H1008_sensitive        GTGAAAGTTACGCAGACTGTCGCCATAGTTGTGTTTTTCATTGCCATTATACTGTCTGGG
ILRAD700_sensitive     GTGAAAGTTCCGCAGACTGTCGCCATAGTTGTGTTTTTCATTGCCATTATACTGTCTGGG
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H65_resistant          ATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGTGTCGT
H89_resistant          ATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGTGTCGT
H988_sensitive         ATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGTGTCGT
H1008_sensitive        ATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGTGTCGT
ILRAD700_sensitive     ATTGGTAAGTCACATATGGAGGCCACCACTTACACGCTGGTGTCGT
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TvY486_0041960F2 *CACTTACACGCTGGTGTCGT*
TvY486_0041960R2 *AGGGCCATATGCACTTGAAC*

CLUSTAL 2.0.12 multiple sequence alignment

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H65_resistant           CACTTACACGCTGGTGTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTG
H89_resistant           CACTTACACGCTGGTGTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTG
H988_sensitive          CACTTACACGCTGGTGTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTG
H1008_sensitive         CACTTACACGCTGGTGTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTG
ILRAD700_sensitive      CACTTACACGCTGGTGTCGTCCATGCCGTCAAAGTTCATGTCCGCTGCCATGTTTGGCTG
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H65_resistant           CTCGTTCTCTGGCGTGATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H89_resistant           CTCGTTCTCTGGCGTGATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H988_sensitive          CTCGTTCTCTGGCGTGATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
H1008_sensitive         CTCGTTCTCTGGCGTGATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
ILRAD700_sensitive      CTCGTTCTCTGGCGTGATAACATCGGTGCTGCAGTGCGTGATCAAGGGGTCGATGGAGAA
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H65_resistant           CACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTGGGTTGGTGATTAT
H89_resistant           CACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTGGGTTGGTGATTAT
H988_sensitive          CACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCCTGGGTTGGTGATTAT
H1008_sensitive         CACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCCTGGGTTGGTGATTAT
ILRAD700_sensitive      CACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCCTGGGTTGGTGATTAT
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H65_resistant           GACCGTGGCGCTCATCATGGCGCACTCGCTGCGCTACATTTCCTACGCACAGGAGAACGT
H89_resistant           GACCGTGGCGCTCATCATGGCGCACTCGCTGCGCTACATTTCCTACGCACAGGAGAACGT
H988_sensitive          GACCGTGGCGCTCATCATGGCGCACTCGCTGCGCTACATTTCCTACGCACAGGAGAACGT
H1008_sensitive         GACCGTGGCGCTCATCATGGCGCACTCGCTGCGCTACATTTCCTACGCACAGGAGAACGT
ILRAD700_sensitive      GACCGTGGCGCTCATCATGGCGCACTCGCTGCGCTACATTTCCTACGCACAGGAGAACGT
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H65_resistant          TGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGGATGCCACAACGACAC
H89_resistant          TGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGGATGCCACAACGACAC
H988_sensitive         TGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGGATGCCACAACGACAC
H1008_sensitive        TGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGGATGCCACAACGACAC
ILRAD700_sensitive     TGCTGAGTACCGCATGATGAAGCAAGCGAATAGTGACGAGGGCGGATGCCACAACGACAC
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H65_resistant          AGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGGGAGTGTAGACGAAGAGGCGGG
H89_resistant          AGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGGGAGTGTAGACGAAGAGGCGGG
H988_sensitive         AGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGGGAGTGTAGACGAAGAGGCGGG
H1008_sensitive        AGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGGGAGTGTAGACGAAGAGGCGGG
ILRAD700_sensitive     AGACGGTGAAAACGAGCCCGTTGCCAAGATGGAGGAGGGGAGTGTAGACGAAGAGGCGGG
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H65_resistant          CATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCCTGTATTAAAGAAAATACA
H89_resistant          CATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCCTGTATTAAAGAAAATACA
H988_sensitive         CATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCCTGTATTAAAGAAAATACA
H1008_sensitive        CATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCCTGTATTAAAGAAAATACA
ILRAD700_sensitive     CATGACAACGGCAGAGCAACTCACGGCAACACCTGTCCTGCCTGTATTAAAGAAAATACA
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H65_resistant          CCTGATGATGACCACGTGCTTCATTTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCT
H89_resistant          CCTGATGATGACCACGTGCTTCATTTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCT
H988_sensitive         CCTGATGATGACCACGTGCTTCATTTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCT
H1008_sensitive        CCTGATGATGACCACGTGCTTCATTTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCT
ILRAD700_sensitive     CCTGATGATGACCACGTGCTTCATTTCCTTCTTTGTCACGCTCTTTATATTCCCGAGTCT
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H65_resistant          TGTCTTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAA
H89_resistant          TGTCTTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAA
H988_sensitive         TGTCTTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAA
H1008_sensitive        TGTCTTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAA
ILRAD700_sensitive     TGTCTTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTACAA
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H65_resistant           CTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCT
H89_resistant           CTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCT
H988_sensitive          CTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCT
H1008_sensitive         CTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCT
ILRAD700_sensitive      CTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCT
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TvY486_0041960F3        TCTTGTCCTTCCCATTGACC
TvY486_0041960R3        AAGGTACTGTTAGGGCAAAGC

CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           TCTTGTCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTA
H89_resistant           TCTTGTCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTA
H988_sensitive          TCTTGTCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTA
H1008_sensitive         TCTTGTCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTA
ILRAD700_sensitive      TCTTGTCCTTCCCATTGACCGAGACCACAACTGGTTTGGCACGCTTGCGATTCTGTGCTA
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H65_resistant           CAACTTTGGCGAGGCCGTTGCCAATTTTGGCACAACGTTCAAGTGCATATGGCCCTCGCG
H89_resistant           CAACTTTGGTGAGGCCGTTGGACGCTTCTCCACAACGTTCAAGTGCATATGGCCCTCGCG
H988_sensitive          CAACTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCTCGCG
H1008_sensitive         CAACTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCTCGCG
ILRAD700_sensitive      CAACTTTGGTGATGCAGCAGGACGCTTTGGCACAACGTTCAAGTGCATATGGCCCTCGCG
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H65_resistant           ACGCGTTTTACTCATCCTCACCCTCTCCCGCTTTCTCTTCATTGTGCCCATCTTTCTCTG
H89_resistant           ACGCGTTTTACTCATCCTCACCCTCTCCCGCTTTCTCTTCATTGTGCCCATCTTTCTCTG
H988_sensitive          ACGCGTTTTACTCATCCTCACCCTCTCCCGCTTTCTCTTCATTGTGCCCATCTTTCTCTG
H1008_sensitive         ACGCGTTTTACTCATCCTCACCCTCTCCCGCTTTCTCTTCATTGTGCCCATCTTTCTCTG
ILRAD700_sensitive      ACGCGTTTTACTCATCCTCACCCTCTCCCGCTTTCTCTTCATTGTGCCCATCTTTCTCTG
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H65_resistant           TGTCTTTAAATACATCCCGGGACATGCAGTACCTTACATTCTCATGTTTCTCGTTGGATT
H89_resistant           TGTCTTTAAATACATCCCGGGACATGCAGTACCTTACATTCTCATGTTTCTCGTTGGATT
H988_sensitive          TGTCTTTAAATACATCCCGGGACATGCAGTACCTTACATTCTCATGTTTCTCGTTGGATT
H1008_sensitive         TGTCTTTAAATACATCCCGGGACATGCAGTACCTTACATTCTCATGTTTCTCGTTGGATT
ILRAD700_sensitive      TGTCTTTAAATACATCCCGGGACATGCAGTACCTTACATTCTCATGTTTCTCGTTGGATT
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H65_resistant           GACGAACTACACGGGTACACTGTCGATGGTGTACGGGCCGATTACGCCTGGGCTTGTGAC
H89_resistant           GACGAACTACACGGGTACACTGTCGATGGTGTACGGGCCGATTACGCCTGGGCTTGTGAC
H988_sensitive          GACGAACTACACGGGTGCACTGTCGATGGTGTACGGGCCGATTACGCCTGGGCTTGTGAC
H1008_sensitive         GACGAACTACACGGGTGCACTGTCGATGGTGTACGGGCCGATTACGCCTGGGCTTGTGAC
ILRAD700_sensitive      GACGAACTACACGGGTGCACTGTCGATGGTGTACGGGCCGATTACGCCTGGGCTTGTGAC
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H65_resistant           TGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATGGGCATTTCTCTGCTTGCTGGTGCCTC
H89_resistant           TGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATGGGCATTTCTCTGCTTGCTGGTGCCTC
H988_sensitive          TGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATGGGCATTTCTCTGCTTGCTGGTGCCTC
H1008_sensitive         TGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATGGGCATTTCTCTGCTTGCTGGTGCCTC
ILRAD700_sensitive      TGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATGGGCATTTCTCTGCTTGCTGGTGCCTC
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H65_resistant           TTTCGCATCGCTGATTGCCATCGGTGTCGCGTACGCTTTGCCCTAACAGTACCTT
H89_resistant           TTTCGCATCGCTGATTGCCATCGGTGTCGCGTACGCTTTGCCCTAACAGTACCTT
H988_sensitive          TTTCGCATCGCTGATTGCCATCGGTGTCGTGTACGCTTTGCCCTAACAGTACCTT
H1008_sensitive         TTTCGCATCGCTGATTGCCATCGGTGTCGTGTACGCTTTGCCCTAACAGTACCTT
ILRAD700_sensitive      TTTCGCATCGCTGATTGCCATCGGTGTCGTGTACGCTTTGCCCTAACAGTACCTT
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Sequencing performed on TvY486_1103760 using 2 resistant and 3 sensitive *T. vivax* strains

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>TvY486_1103760 F2      CGCTACATTTCCTACGCACA
>TvY486_1103760 R2      ATGCCAATGGGCAGTCTTAG

CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           CGCTACATTTCCTACGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAAT
H89_resistant           CGCTACATTTCCTACGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAAT
H988_sensitive          CGCTACATTTCCTACGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAAT
H1008_sensitive         CGCTACATTTCCTACGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAAT
ILRAD700_sensitive      CGCTACATTTCCTACGCACAGGAGAACGTTGCTGAGTACCGCATGATGAAGCAAGCGAAT
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H65_resistant           AGTGACGAGGGCGGATGCCACAACGACACAGACGGTGAAAACGAGCCCGTTGCCAAGATG
H89_resistant           AGTGACGAGGGCGGATGCCACAACGACACAGACGGTGAAAACGAGCCCGTTGCCAAGATG
H988_sensitive          AGTGACGAGGGCGGATGCCACAACGACACAGACGGTGAAAACGAGCCCGTTGCCAAGATG
H1008_sensitive         AGTGACGAGGGCGGATGCCACAACGACACAGACGGTGAAAACGAGCCCATTGCCAAGATG
ILRAD700_sensitive      AGTGACGAGGGCGGATGCCACAACGACACAGACGGTGAAAACGAGCCCATTGCCAAGATG
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H65_resistant           GAGGAGGGGAGTGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACA
H89_resistant           GAGGAGGGGAGTGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACA
H988_sensitive          GAGGAGGGGAGTGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACA
H1008_sensitive         GAGGAGGGGAGTGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACA
ILRAD700_sensitive      GAGGAGGGGAGTGTAGACGAAGAGGCGGGCATGACAACGGCAGAGCAACTCACGGCAACA
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H65_resistant           CCTGTCCTGCCTGTATTAAAGAAAATACACCTGATGATGACCACGTGCTTCATTTCCTTC
H89_resistant           CCTGTCCTGCCTGTATTAAAGAAAATACACCTGATGATGACCACGTGCTTCATTTCCTTC
H988_sensitive          CCTGTCCTGCCTGTATTAAAGAAAATACACCTGATGATGACCACGTGCTTCATTTCCTTC
H1008_sensitive         CCTGTCCTGCCTGTATTAAAGAAAATACACCTGATGATGACCACGTGCTTCATTTCCTTC
ILRAD700_sensitive      CCTGTCCTGCCTGTATTAAAGAAAATACACCTGATGATGACCACGTGCTTCATTTCCTTC
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H65_resistant          TTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCACAACTGG
H89_resistant          TTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCACAACTGG
H988_sensitive         TTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCACAACTGG
H1008_sensitive        TTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCACAACTGG
ILRAD700_sensitive     TTTGTCACGCTCTTTATATTCCCGAGTCTTGTCTTTCCCATTGACCGAGACCACAACTGG
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H65_resistant          TTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTTTGGCACA
H89_resistant          TTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTTTGGCACA
H988_sensitive         TTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTTTGGCACA
H1008_sensitive        TTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTTTGGCACA
ILRAD700_sensitive     TTTGGCACGCTTGCGATTCTGTGCTACAACTTTGGTGATGCAGCAGGACGCTTTGGCACA
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H65_resistant          ACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTCCCGCTTT
H89_resistant          ACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTCCCGCTTT
H988_sensitive         ACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCGCCCTCTCCCGCTTT
H1008_sensitive        ACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTCCCGCTTT
ILRAD700_sensitive     ACGTTCAAGTGCATATGGCCCTCGCGACGCGTTTTACTCATCCTCACCCTCTCCCGCTTT
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H65_resistant          CTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGCAGTACCT
H89_resistant          CTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGCAGTACCT
H988_sensitive         CTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGCAGTACCT
H1008_sensitive        CTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGCAGTACCT
ILRAD700_sensitive     CTCTTCATTGTGCCCATCTTTCTCTGTGTCTTTAAATACATCCCGGGACATGCAGTACCT
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H65_resistant          TACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGATGGTGTAC
H89_resistant          TACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGATGGTGTAC
H988_sensitive         TACATTCTCACGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGATGGTGTAC
H1008_sensitive        TACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGATGGTGTAC
ILRAD700_sensitive     TACATTCTCATGTTTCTCGTTGGATTGACGAACTACACGGGTGCACTGTCGATGGTGTAC
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H65_resistant          GGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATG
H89_resistant          GGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATG
H988_sensitive         GGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATG
H1008_sensitive        GGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATG
ILRAD700_sensitive     GGGCCGATTACGCCTGGGCTTGTGACTGCAGGGCAGAAGCTGATGGCCGGGCAGCTGATG
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H65_resistant           GGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGTCGTGTAC
H89_resistant           GGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGTCGTGTAC
H988_sensitive          GGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGTCGTGTAC
H1008_sensitive         GGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGTCGTGTAC
ILRAD700_sensitive      GGCATTTCTCTGCTTGCTGGTGCCTCTTTCGCATCGCTGATTGCCATCGGTGTCGTGTAC
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H65_resistant           GCTTTGCCCTAAGACTGCCCATTGGCAT
H89_resistant           GCTTTGCCCTAAGACTGCCCATTGGCAT
H988_sensitive          GCTTTGCCCTAAGACTGCCCATTGGCAT
H1008_sensitive         GCTTTGCCCTAAGACTGCCCATTGGCAT
ILRAD700_sensitive      GCTTTGCCCTAAGACTGCCCATTGGCAT
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Sequencing performed on TvY486_0011610 using 2 resistant and 3 sensitive *T. vivax* strains

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TvY486_0011610 F1       CAAATATGCTCCTGGGCTTCT
TvY486_0011610 R1       ATGACAAGTGAGGCGGCTAT

CLUSTAL 2.0.12 multiple sequence alignment


H65_resistant           CAAATATGCTCCTGGGCTTCTCCTCGGCCGCAGAACTGTACACGTATGTGACGTGCGTTA
H89_resistant           CAAATATGCTCCTGGGCTTCTCCTCGGCCGCAGAACTGTACACGTATGTGACGTGCGTTA
H988_sensitive          CAAATATGCTCCTGGGCTTCTCCTCGGCCGCAGAACTGTACACGTATGTGACGTGCGTTA
H1008_sensitive         CAAATATGCTCCTGGGCTTCTCCTCGGCCGCAGAACTGTACACGTATGTGACGTGCGTTA
ILRAD700_sensitive      CAAATATGCTCCTGGGCTTCTCCTCGGCCGCAGAACTGTACACGTATGTGACGTGCGTTA
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H65_resistant           TCCTTGGCATTTCGTTTCTCCTGCCGCTTAAAGTCATGGTATCTGCACCGCGCTTTATGA
H89_resistant           TCCTTGGCATTTCGTTTCTCCTGCCGCTTAAAGTCATGGTATCTGCACCGCGCTTTATGA
H988_sensitive          TCCTTGGCATTTCGTTTCTCCTGCCGCTTAAAGTCATGGTATCTGCACCGCGCTTTATGA
H1008_sensitive         TCCTTGGCATTTCGTTTCTCCTGCCGCTTAAAGTCATGGTATCTGCACCGCGCTTTATGA
ILRAD700_sensitive      TCCTTGGCATTTCGTTTCTCCTGCCGCTTAAAGTCATGGTATCTGCACCGCGCTTTATGA
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H65_resistant          CCGACTACTACAAGTACGCAACTGGTGACCCGGATGCCGAGCCGAACAATCCTTTTTTTT
H89_resistant          CCGACTACTACAAGTACGCAACTGGTGACCCGGATGCCGAGCCGAACAATCCTTTTTTTT
H988_sensitive         CCGACTACTACAAGTACGCAACTGGTGACCCGGATGCCGAGCCGAACAATCCTTTTTTTT
H1008_sensitive        CCGACTACTACAAGTACGCAACTGGTGACCCGGATGCCGAGCCGAACAATCCTTTTTTTT
ILRAD700_sensitive     CCGACTACTACAAGTACGCAACTGGTGACCCGGATGCCGAGCCGAACAATCCTTTTTTTT
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H65_resistant          GGGCAAATGTCCTCGGTATTTACGCCGCGGCGTCGCTGGTTGTACAAATGCTCTTCGCCC
H89_resistant          GGGCAAATGTCCTCGGTATTTACGCCGCGGCGTCGCTGGTTGTACAAATGCTCTTCGCCC
H988_sensitive         GGGCAAATGTCCTCGGTATTTACGCCGCGGCGTCGCTGGTTGTACAAATGCTCTTCGCCC
H1008_sensitive        GGGCAAATGTCCTCGGTATTTACGCCGCGGCGTCGCTGGTTGTACAAATGCTCTTCGCCC
ILRAD700_sensitive     GGGCAAATGTCCTCGGTATTTACGCCGCGGCGTCGCTGGTTGTACAAATGCTCTTCGCCC
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H65_resistant          CCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGCTTCACCTTTGCCG
H89_resistant          CCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGCTTCACCTTTGCCG
H988_sensitive         CCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGCTTCACCTTTGCCG
H1008_sensitive        CCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGCTTCACCTTTGCCG
ILRAD700_sensitive     CCACAGTTCTCACTCGCACGGTCAGGCGCCTCTCCTTGAGTACGCGCTTCACCTTTGCCG
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H65_resistant          TCTCGTCCATGCTGGCTGGGGCGGCAGTTATCCCTCTGATGCCTGTAGTGAAAGTTACGC
H89_resistant          TCTCGTCCATGCTGGCTGGGGCGGCAGTTATCCCTCTGATGCCTGTAGTGAAAGTTACGC
H988_sensitive         TCTCGTCCATGCTGGCTGGGGCGGCAGTTATCCCTCTGATGCCTGTAGTGAAAGTTACGC
H1008_sensitive        TCTCGTCCATGCTGGCTGGGGCGGCAGTTATCCCTCTGATGCCTGTAGTGAAAGTTACGC
ILRAD700_sensitive     TCTCGTCCATGCTGGCTGGGGCGGCAGTTATCCCTCTGATGCCTGTAGTGAAAGTTACGC
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H65_resistant          AGACTGTCGCAATGGTTGTGCTCTTTGTCTCCATTTTCCTCTCTTCGATGGGAAAGGCGT
H89_resistant          AGACTGTCGCAATGGTTGTGCTCTTTGTCTCCATTTTCCTCTCTTCGATGGGAAAGGCGT
H988_sensitive         AGACTGTCGCAATGGTTGTGCTCTTTGTCTCCATTTTCCTCTCTTCGATGGGAAAGGCGT
H1008_sensitive        AGACTGTCGCAATGGTTGTGCTCTTTGTCTCCATTTTCCTCTCTTCGATGGGAAAGGCGT
ILRAD700_sensitive     AGACTGTCGCAATGGTTGTGCTCTTTGTCTCCATTTTCCTCTCTTCGATGGGAAAGGCGT
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H65_resistant          ATTTGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGGTG
H89_resistant          ATTTGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGGTG
H988_sensitive         ATTTGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGGTG
H1008_sensitive        ATTTGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGGTG
ILRAD700_sensitive     ATTTGGAGGCCACCACTTACACGCTGGTGTCGTCCATGCCGCCAAAGTTCATGTCCGGTG
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H65_resistant           CCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAAGG
H89_resistant           CCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAAGG
H988_sensitive          CCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAAGG
H1008_sensitive         CCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAAGG
ILRAD700_sensitive      CCATGTTTGGTGCCTCGCTCTGTGGCGTGATAACGTCGGTGCTGCAGTGCGTGATCAAGG
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H65_resistant           GGTCGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTG
H89_resistant           GGTCGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTG
H988_sensitive          GGTCGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTG
H1008_sensitive         GGTCGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTG
ILRAD700_sensitive      GGTCGATGGAGAACACATATGAGTCTGTACTGAAGCAGTCGTACATCTACTTCTCCCTTG
                        ************************************************************
H65_resistant           GGATGGTGATTATAGCCGCCTCACTTGTCAT
H89_resistant           GGATGGTGATTATAGCCGCCTCACTTGTCAT
H988_sensitive          GGATGGTGATTATAGCCGCCTCACTTGTCAT
H1008_sensitive         GGATGGTGATTATAGCCGCCTCACTTGTCAT
ILRAD700_sensitive      GGATGGTGATTATAGCCGCCTCACTTGTCAT
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